Optimisation and Application of Comparative Genomic Hybridisation (CGH) In Cancer Cytogenetics

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To my husband Young Tae Kim
And my children Min-Young and Yong-Hee

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I have incurred my debts and sacrifices from the people around me while studying this Ph D course.

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Kim MH, Stewart J, Devlin C, Boyd E, Connor JM (1998): Detection of Hyperdiploidy in Acute Lymphoblastic Leukaemia by Comparative Genomic Hybridization. J Med Genet 35: (2.22)

Kim MH, Stewart J, Devlin C, Boyd E, Connor JM (1998): Identification of the Origin of Marker Chromosomes by CGH and In Situ Hybridisation in Patients with AML and MDS. J Med Genet 35: (12.12)

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LIST of ABBREVIATIONS

A	Adenine
ABL	Abelson
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BCR	breakpoint cluster region
bp	base pair
C	Cytosine
°C	Degree of Celsius
CCD	Charge Coupled Device
CDK	cyclin-dependent kinase
CGH	Comparative Genomic Hybridization
CISS	Chromosomal in situ suppression
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
cm	Centimeter
Cot	Concentration over time
DAPI	4′-6-diamino-2-phenylindole
del	Deletion
dic	Dicentric
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate

dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTPs	Deoxyribonucleotide triphosphate
DMS	Double minutes
DNA	Deoxyribonucleic acid
DOP-PCR	Degenerate Oligonucleotide Primed PCR
dup	Duplication
EDTA	Ethylenediamine tetraacetic acid
e.g.	exemplia gratia (for example)
EHF PCR system	Expand High Fidelity Polymerase Chain Reaction system
et al.	et alia
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein isothiocyanate
G	Guanine
G_1	first gap phase of cell cycle
G_2	second gap phase of cell cycle
g	gram
G-banding	Giemsa banding
HSRs	Homogeneously stained regions
ISH	In Situ Hybridization
inv	Inversion
kb	kilobase
LINEs	Long Interspersed Nuclear Elements
LOH	loss of heterozygosity

M	Molar
mar	Marker chromosome
Mb	Megabase
MDM2	murine double minute 2
MDS	myelodysplastic syndromes
MPD	Myeloproliferative disorders
μg	Microgram
μl	Microlitre
μM	Micro molar
mg	Miligram
ml	Mililitre
mm	Milimetre
mM	Mili molar
ng	nanogram
NHL	non-Hodgkin's lymphoma
OD	Optical Density
P	Short arm of chromosome
PCR	polymerase chain reaction
pg	pecogram
PHA	Phytohaemagglutinin
PCR	Polymerase Chain Reaction
PEP	Primer extension preamplification
pmol	Picomol
q	Long arm of chromosome

r	Ring chromosome
RNA	Ribonucleic acid
ROI	Region of interest
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SINEs	Short Interspersed Nuclear Elements
SSC	Saline sodium citrate
T	Thymine
t	Translocation
Tm	Melting temperature
TBE	Tris-acetate ethylenediaminetetra acetic acid
TE	Tris-ethylenediaminetetra acetic acid
ter	Terminal
UV	Ultraviolet
V/V	Volume per volume
WCP	Whole chromosome painting
WDLPS	Well differentiated liposarcoma
YAC	Yeast artificial chromosome

SUMMARY

The aim of this study was to assess to the applicability of the CGH technique to analysis of various kinds of malignancies in particular hematological malignancies and solid tumours, and to optimise a CGH technique for use on very small amounts of DNA from paraffin embedded tissue.

During the initial part of the study several FISH experiments were also performed. First, interphase FISH with selected chromosome specific alpha satellite probes was applied to 22 cases with acute lymphoblastic leukemia, to investigate whether this could contribute to the detection of hidden hyperdiploidy or detect aneuploidy in cases where cytogenetic analysis had failed. Then CGH and interphase FISH were both performed in 19 cases with acute lymphoblastic leukemia to compare the efficiency of the two approaches. CGH showed potential as a comprehensive screening method for detection of hyperdiploid cases and additionally unbalanced DNA copy number changes including one amplification on 6p23-pter were detected. Interphase FISH also might be a complementary method to confirm CGH results, especially in hyperdiploid or hypotriploid cases which failed by conventional karyotyping.

CGH analysis in twenty-two cases of AML, MDS or MDS in transformation, revealed that net gains and losses of chromosomal material could be detected more simply and accurately by CGH than by conventional karyotyping in cases with a complex karyotype. CGH followed by FISH using specific probes which were chosen according to discordance between CGH and conventional karyotyping could identify the origin of marker chromosomes, and CGH also could detect amplification sites where candidate genes related to pathogenesis of cancer might be harbored.

A further fourteen cases of other hematological malignancies were analysed by CGH and another hidden abnormality which was missed by conventional karyotyping was detected in 1 case.

Next, CGH experiments moved to fresh frozen solid tumours. Five cases of well differentiated liposarcoma and seventeen cases of malignant melanoma were analysed. In well differentiated liposarcoma CGH could detect additional amplifications including one amplification (3q12-q13.3) which was hitherto unreported and helped to evaluate the composition of marker chromosomes. In cases with malignant melanoma, CGH could identify

frequently involved regions of gains and losses, the inter-relationship between abnormalities, and found three new sites of amplification (1p11.1-p12, 3p24, and 22) and amplification on 17q24-qter in malignant melanoma.

The CGH technique was next applied to very small amounts of DNA from paraffin-embedded tissue of solid tumours. For success of this experiment, several steps from the extraction of DNA to CGH itself had to be optimised. Although it would have been ideal to move to the next step after completing the optimisation of one step, most experiments involving the extraction of DNA went on to attempt CGH because these test samples were precious material and the final goal of this experiment was to obtain successful CGH result.

For obtaining an adequate quantity of high quality of DNA, fifteen different parameters were investigated to discover the optimal method for extraction of DNA from paraffin-embedded tissue. According to this optimisation process, in a very small sample (1 x 7μm) a single step cell lysis method (without phenol/chloroform extraction) and higher dosage of proteinase K (1-2 mg/ml) with shorter duration (≤ 24 hrs) showed better results.

For whole genome amplification, two methods – primer extension preamplification (PEP) and degenerate oligonucleotide primed-PCR (DOP-PCR) were used. DOP-PCR was performed with 8 different minor modifications. The DOP-PCR method without disruption from start to finish was the better method for avoidance of contamination and cases using thermosequenase showed better results than those using combination of topoisomerase and T7 sequenase. PEP was performed with several modifications to assess if whole genome amplification (WGA) using PEP could produce analysable CGH images and if so what volume of starting template DNA could produce CGH images by this method. Another aim was to evaluate the difference of results in comparison with DOP-PCR. Although PEP produced some successful CGH images, it showed some limitation to amplify a sufficient amount of DNA to produce reliable CGH from very small samples. By comparing results between both methods, there are some discrepancies of amplification pattern.

After the optimisation process was almost completed, this technique was applied to three kinds of serially diluted samples (DNA from normal female blood, abnormal bone marrow with AML/MDS, and thick-sectioned paraffin-embedded ovarian cancer tissue).

These experiments showed that the smallest amount of template DNA which could produce reliable CGH images was above10 pg of DNA from normal

female blood, above 50 pg of DNA from abnormal bone marrow with complex chromosome aberrations, and above 100 pg of DNA from thick sectioned paraffin-embedded ovarian cancer tissue. This study also revealed that the application of the same amplification and labelling method to both test and control DNA was the ideal method for decreasing bias developed during the amplification and labelling process. The experiments with proofreading enzyme showed an increase of sensitivity and reliability.

Finally, this optimised technique was employed to unknown malignant melanoma samples. Five cases were studied. In the first experiment one whole section of 7 μm thickness was analysed from each, and in the second experiment microdissected samples from the sections of different thickness (3 μm and 7 μm) from each case were studied. In four of the five cases, constant abnormal findings were observed. The fifth case failed to obtain proper result because of insufficient amount of sample.

For validation of these results, inverse labelling of CGH, tissue in situ hybridisation, and single round PCR with specific sequence of primers were performed.

CGH could not detect 100% of abnormal copy numbers, especially loss of DNA copy number, in experiments using very small amounts of DNA. It is thought that this is caused by limits of resolution of CGH for detecting deletion, problems in the samples themselves (e.g. contamination of normal cells), and some bias developed during the amplification process. However, in spite of these limitations and the difficulties encountered during the whole optimisation process, it can be concluded from the work described here that reliable CGH studies can indeed be undertaken on amplified DNA obtained from a very small amount of paraffin-embedded tissue.

CHAPTER 1.

INTRODUCTION

1. INTRODUCTION

1.1 General Introduction

Although diseases of the heart and blood vessels are still the main cause of death especially in an aging population, cancer has become one of the most common causes of death in humans today due to increased environmental hazards such as cigarette smoking, exposure to chemicals and radiation, and prolonged life expectancy. Accumulation of genetic alterations are at the very centre of tumorigenesis so that at a cellular level cancer can be designated a genetic disorder. To identify the cancer-associated gene mutations is very important to understanding the genetic pathology of cancer. Although other molecular techniques are important in the discovery of the numerous genes that contribute to tumorigenesis, cytogenetic study is also essential to obtain a comprehensive view of cancer genetics. Identification of chromosomal aberrations is having an increasingly important role in clinical management of cancer patients because they can provide critical information for diagnosis and prognosis. The discovery of the Philadelphia chromosome in chronic myeloid leukemia by Nowell and Hungerford in 1960 gave the first direct evidence of a constant chromosomal aberration in a tumour.

Although classical cytogenetics still represents the major tool for evaluation of the chromosomal abnormalities, it has some limitations in the resolution of complex karyotypes with multiple chromosomal rearrangements. To facilitate more accurate definition of rearrangements as well as the identification of previously undetected chromosomal aberrations, various molecular cytogenetic techniques such as FISH, multi-colour FISH, and CGH are developing as a complementary method to aid more accurate comprehensive genetic analysis, especially in cancer genetics.

The role of cytogenetics within modern biology is continually changing and developing by adding molecular technology, and modern computer science, and

thus the value of cytogenetics is becoming more important in the field of cancer genetics.

1.2 DNA

1.2.1 The double helix

DNA is a heteropolymer composed of a chain of nucleotides. Each nucleotide consists of a sugar molecule (deoxyribose), a phosphate group and one of four bases: a purine (adenine or guanine) or a pyrimidine (cytosine or thymine). The sugar/phosphate components of the nucleotide are important in determining the structural characteristics of polynucleotides, with the nitrogenous bases determining their information storage and transmission characteristics.

A single stranded DNA is composed of the sugar-phosphate backbone structure of the DNA molecule lying on the outside of the DNA fibre with the bases projected inward from the backbone. The free 3′ and 5′ ends as a result of the phosphodiester linkage between their sugars give the molecule a polarity.

To form the double helix, two complementary strands of DNA with opposite polarities pair with each other. The pairing attraction holds by the formation of hydrogen bonds between the purines and pyrimidines (A-T, G-C).

Among the conformations constructed by the double-stranded DNA molecule, the most common form is a right-handed double helix (B-form). There are 10 base pairs in each turn of the helix and both strands are coiled in the same direction.

The stability of the double helical structure of DNA is maintained by stacking forces and by hydrogen bonds between two bases. G-C pairs are more stable than A-T pairs because G-C pairs have three hydrogen bonds whereas A-T pairs have only two hydrogen bonds.

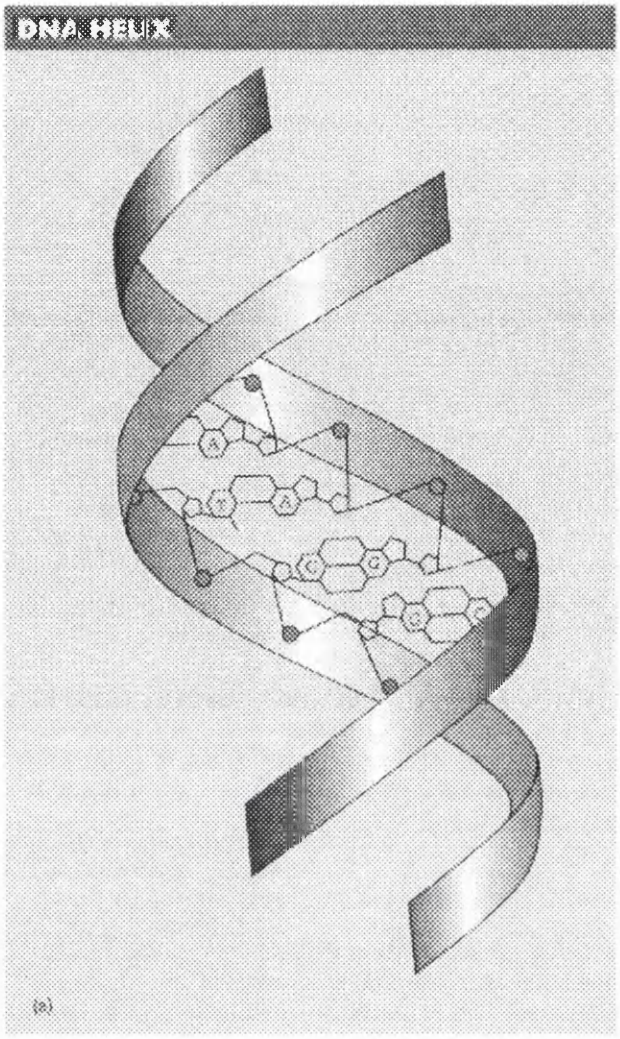


Fig 1.1 DNA double helical structure (adopted from Bradley et al; Lecture notes on molecular medicine, 1995)

1.2.2 The stream of gene expression of genetic information

The human haploid genome contains approximately 3x 10^9 bp of DNA containing approximately 50000 to 100000 different genes. A linear arrangement of codons that carries the information needed to encode a specific polypeptide is known as a gene. The stream of genetic information is unidirectional, from DNA to protein, with messenger RNA as an intermediate. During the expression of a gene, the segment of DNA is first transcribed into a single stranded mRNA, and

then translated from RNA into protein. Proteins, particularly enzymes that catalyze the reaction of metabolism, manifest the final expression of genetic information (Bradley et al., 1995; Nicholl, 1996).

1.2.3 DNA constitution

DNA in both the form of base composition and the type of DNA varies from organism to organism. DNA can be divided into three categories, depending on their abundance in the genome.

1) Single or low-copy DNA.

Single copy genes and their related regulatory sequences occupy approximately 65-70% of total DNA. It contains most of the transcribed genes, but also untranscribable material.

2) Repetitive DNA

a) Highly repetitive DNA is composed of satellite DNA and interspersed DNA. Satellite DNA consists of simple sequences repeated many thousand times and is frequently non-coding. This satellite DNA consists of three types of repetitive sequences- satellite, minisatellite (telomeric family and hypervariable family) and microsatellite. Interspersed repetitive DNAs are found scattered throughout the genome such as short and long repetitive sequences (SINES and LINES). Some of this is transcribed and some of it consists of elements that are present in many genes. One of the most common SINES is the Alu family.

b) Middle (moderately) repetitive DNA are sequences which code for most of the structural components of a cell such as histones, ribosomal RNA and transfer RNAs (Lichter and Cremer, 1992; Raskó and Downes, 1995; Clark and Wall, 1996).

1.2.4 Cot curve

The curve of the rate at which denatured DNA becomes a double helical structure is called the Cot curve.

The Cot analysis of DNA by hybridisation is based on the fact that DNA denatured by extremes of temperature and pH will reanneal with their complementary sequences to form stable double helices under optimal conditions.

The rate of hybridisation depends on the Cot factor, the production of the Co x t. where

Co= initial concentration of DNA in moles of nucleotide per litre.

T= renaturation time in seconds.

If the DNA is fragmented into small,i.e. 400-600bp pieces, and denatured, the highly repetitive sequences will reanneal first because there is a greater probability of complementary sequences encountering each other. The low-copy or single copy sequences will reanneal finally because they take longer to find their complementary strand. The more complex the genome, the longer the time will be needed to find their complementary strands (Fig1.2) (Raskó and Downes, 1995; Clark and Wall, 1996).

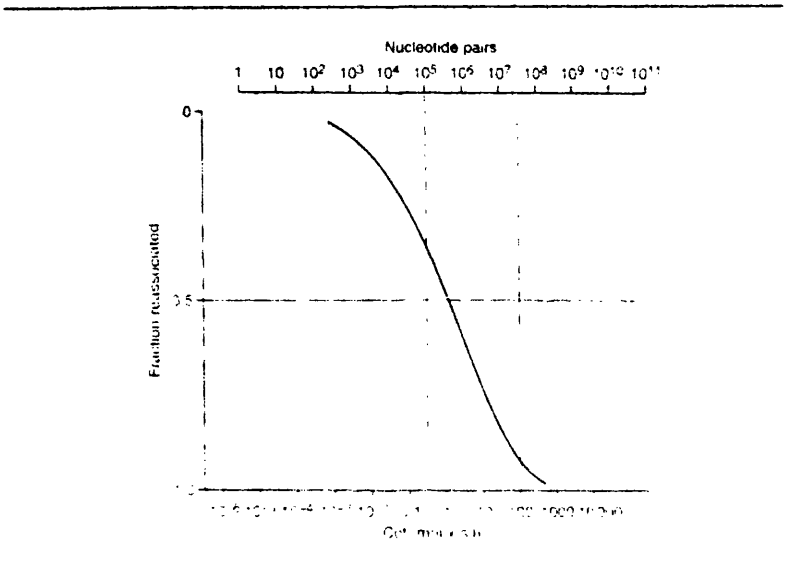


Fig 1.2 A Cot curve (adopted from Clark and Wall; Chromosome, 1996)

1.3 Chromosome

In humans the normal cell nucleus contains 46 chromosomes (23 pairs). One chromosome of each pair is of paternal origin and the other is of maternal origin, and there are 22 pairs of autosomes and 1 pair of sex chromosomes (XX or XY). During each cell cycle the DNA within the chromosomes duplicates itself and the cell then divides into two daughter cells each identical to the parental cell.
By light microscopy, the nucleus appears as an amorphous network of variably condensed fibers not distinguishable as individual entities. This material is called chromatin, which is a nucleoprotein complex. In cell division, the chromatin condenses into deeply staining threadlike or rod-like structures called chromosomes, which are present in specific numbers in each cell of a given species. A metaphase chromosome consists of two symmetrical chromatids which contain a single, uninterrupted and highly folded molecule of DNA. The metaphase chromosome is the most compact form of DNA which can be seen under the light microscope during mitotic cell division.

1.3.1 Chromosome architecture

The total length of DNA in a diploid mammalian cell if extended would be two metre. Therefore each human chromosome is composed of between 1.4 and 7.3 cm of DNA (average 5 cm of DNA), packaged into a few micrometers of chromatid (average 5 um). This constitutes a packing ratio of 1: 10000, most of which results from the interaction of the DNA-chromatin proteins.
At the first level of packing, the DNA is wound around histone disks, so-called nucleosomes, which are the fundamental unit of chromatin. Each disk or bead is an octamer of four core histone proteins (two each of H2A, H2B, H3, and H4) around which a strand of DNA containing 146 bp is wound one and three-quarter times. DNA linkers of 20-100 nucleotide pairs exist between the successive nucleosome cores, interacted rather less strongly with H1. The linker length varies depending on the kind of tissue from which the chromatin is extracted

(Clark and Wall, 1996; Jackson, 1996).

At the second level of folding, interactions between H1 histones and nucleosomes induce the formation of higher-order structures. One of these is the solenoid structure, a fiber 30 nm in diameter consisting of helical arrangement with 6-7 nucleosomes per turn. The exact structure of the 30-nm strand has not yet been established (Therman and Susman, 1993).

The next order of packing is a loop structure. The solenoid fibers form a series of loops, averaging typically 60-100 kb in length. They are held together at the bases of each loop by nuclear matrix which consists of nonhistone protein.

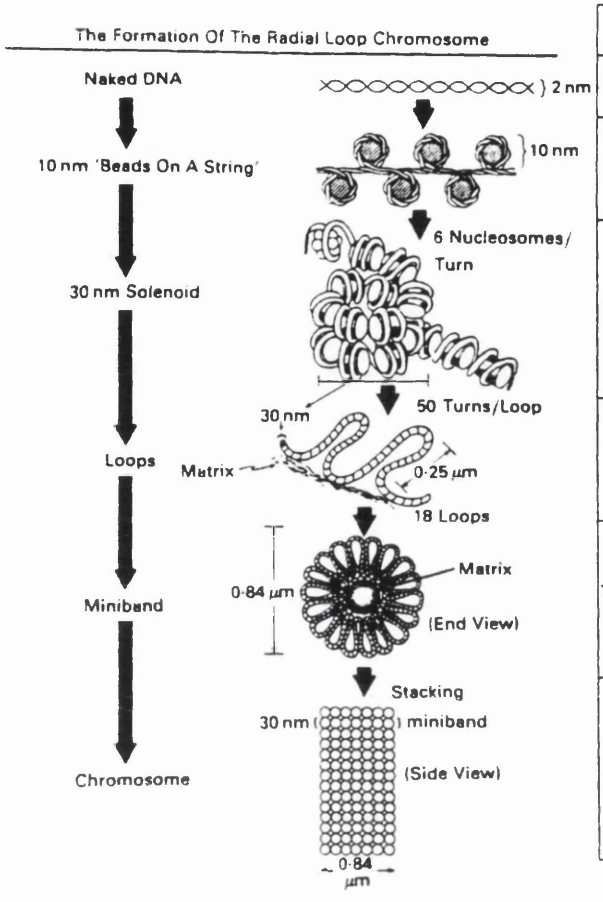
At last, these loops are arranged into rosettes, consisting of a total of 300kb of DNA, producing a 200-300 nm fiber which is the form of the decondensed interphase chromosome. Then these loops are further packed into coils, with each coil corresponding to an average human G-band (approximately 9 Mb of DNA). These coils are then further coiled or stacked to achieve the final metaphase chromosome structure. The number of coils varies according to the amount of DNA in each chromosome (Fig 1.3) (Raskó and Downes, 1995; Clark and Wall, 1996; Jackson, 1996).

1.3.2 Basic structures in a chromosome

These include euchromatin, heterochromatin, centromeres, kinetochores, and telomeres. Chromosomes are composed of two major classes of DNA-euchromatin and heterochromatin.

Euchromatin

Euchromatin is defined as that part of the chromosome which is not heterochromatin, it contains the majority of the active genes and makes up the great majority of the chromosome material in humans. It is believed to be less contracted than the heterochromatin which is enriched with repetitive sequences and highly contracted.



Base-pairs per turn	Packing ratio
10 bp	1
80 bp	6–7
1200 bp	40 ±
60 000 bp	680
$1{\cdot}1 \times 10^6$ bp ±	$1{\cdot}2 \times 10^4$
18 loops/ miniband	$1{\cdot}2 \times 10^4$

Fig 1.3 Hypothetical different level of packing of DNA (adopted from Jackson, chromatin structure and nuclear function: In Molecular biology for oncologists, 1996)

Heterochromatin

Heterochromatin can be divided into constitutive and facultative. Facultative heterochromatin is basically composed of inactivated and condensed euchromatic DNA. One of the well-known examples of facultative heterochromatin is the inactive X in the mammalian female which is condensed in interphase, shows no transcription and is replicated late during the S period. The mechanism bringing about the characteristics of facultative heterochromatin is still unknown (Therman and Susman, 1993).

Constitutive heterochromatin does not contain any unique sequences and has no apparent coding potential. There is considerable variation between individuals in both the amount and distribution of heterochromatin which tends to be concentrated near the centromeres of all the chromosomes, the satellite and short-arm regions of the acrocentric chromosomes, and at the distal end of the Y chromosome (Therman and Susman, 1993; Bradley et al., 1995; Clark and Wall, 1996). It consists of simple repeated sequence DNA. The variation which is found in all the centromeric regions can most easily be visualized in the large blocks of heterochromatin present in chromosomes 1, 9, and 16 by the C-banding technique. Constitutive heterochromatin is not tissue–specific but is individual–specific.

Centromeres

The centromere is the point at which the two chromatids of the metaphase chromosome are held together. It is also the site of the kinetochore, the point of attachment to the spindle fibre during nuclear division.

Centromeric DNA in human chromosomes is largely made up of long tandem repeats of a basic monomeric sequence that is approximately 170 bp (Choo et al., 1991). These are alphoid satellite repeats which represent approximately 0.75% of the human genome or 3-5% of each chromosome (Choo et al., 1991; Bartch and Schwinger, 1991). The repeat sequences in centromeres are often highly

conserved between the chromosomes (Bartch and Schwinger, 1991). Among at least 33 different alphoid subfamilies which have been already identified, some of these families are specific for a single chromosome, while others are common to a small group of chromosomes. In other words, some chromosomes appear to have only a single subfamily within their centromeres, whereas other chromosomes may consist of several different subfamilies (Choo et al., 1991).
A GGAAT repeat has been found to be extremely common and an AT repeat also has been found frequently at centromeres. Although the total length of the centromeric DNA is different from chromosome to chromosome, all centromeres are very large, ranging from about 300 to 5000 kbp. The size of the centromeric heterochromatin is polymorphic and is important to maintain chromosome integrity (Clark and Wall, 1996).

Telomeres

Normal chromosome ends are capped by so-called telomeres which consist of both protein and DNA. These DNA sequences are an essential component in the control of chromosome integrity. Telomeres prevent normal chromosome ends from joining each other, whereas broken ends losing telomeres often tend to fuse end to end, leading to dicentric, ring or other unstable chromosome forms.
It seems that normal chromosome duplication brings on progressive shortening of telomeres after a number of cell divisions, eventually the chromosomes start to lose genetic material and the cell dies. The function of the telomeres to prevent shortening of the chromosomes after each round of cell division is brought about as a result of the action of the enzyme telomerase.
In human chromosomes, the sequence motif of telomere is TTAGGG (Moyzis et al., 1988) repeated over and over to make a strand of DNA around 10 kilobase (kb) pairs long in sperm and 15kb long in somatic cells at the end of each chromosome. This repeated sequence has a highly conserved nature (Clark and Wall, 1996).
DNA at the extreme ends of telomeres is in the form not of a double helix but a single G-rich strand which protrudes beyond the complementary C-rich strand

by formation of the hairpin structure necessary for telomere integrity.

Telomerase is an enzyme for replicaton of telomeres which acts by adding new repeat units to the 3′-end of the G-rich strand. This enzyme is a reverse transcriptase, synthesizing DNA from an RNA template, which may have a tissue–specific activity level (Blackburn, 1991). Defective telomerase activity in cells results in progressive shortening of their chromosomes, senescence, and death.

In tumor cells, chromosome breaks and cryptic translocations may be able to stabilize by the capture of telomere repeats from another chromosome. Although it is not yet discovered how much is causal and how much is secondary, this could be a major explanation of the etiology of the chromosome 6 involvement in melanoma (Meltzer et al., 1993; Clark and Wall, 1996). The activation of telomerase might be associated with preventing cell senescence and death by chromosome loss in tumour cells, for example ovarian cancer (Clark and Wall, 1996).

1.4 Nuclear division and Cell cycles

1.4.1 Nuclear division

In order to be transmitted to the next generation, parental genes have to duplicate their genetic material and transfer the copies to the offspring. The genetic information existing in each cell is transferred during cell division, both in mitosis and meiosis. Mitosis, which occurs in the division of somatic (non-reproductive) cells, results in accurate reproduction in two daughter cells from the chromosomes of the parental cell. Meiosis or reduction division occurs only in the formation of gametes. The number of chromosomes in each daughter cell is reduced to the haploid number.

Mitosis

The average duration of the mitotic cell cycle is 24 hours. Each chromosome replicates itself during the S stage of the cell cycle, and one copy of each chromosome is transferred to each of the two daughter cells during mitosis. Mitosis can be divided into five stages: prophase, prometaphase, metaphase, anaphase, and telophase (Fig 1.4).

i) Prophase

The chromosomes appear as long thin threads. In middle to late prophase the chromosomes are changing gradually to a shortened and thickened state to form chromatids. During prophase, the nucleolus cannot be detected under the light microscope. In the cytoplasm, the centrioles begin to move to opposite poles of the cell and to form the microtubules that make up the mitotic spindle.

ii) Prometaphase

This stage is between prophase and metaphase. The nuclear membrane disperses and disappears, releasing the chromosomes into the cytoplasm. The chromosomes show nearly maximum condensation, and collect on the metaphase plate. At this time the spindle fibres are not yet attached to the kinetochore to which they later anchor for chromatid separation.

iii) Metaphase

At metaphase, the centrioles are in place at opposite poles and the mitotic spindle is accomplished. The chromosomes become aligned at the metaphase plate with the spindle fiber attached to each kinetochore, and the kinetochores are facing opposite poles of the cell. At metaphase, the chromosomes become the most condensed state and are also metabolically inactive.

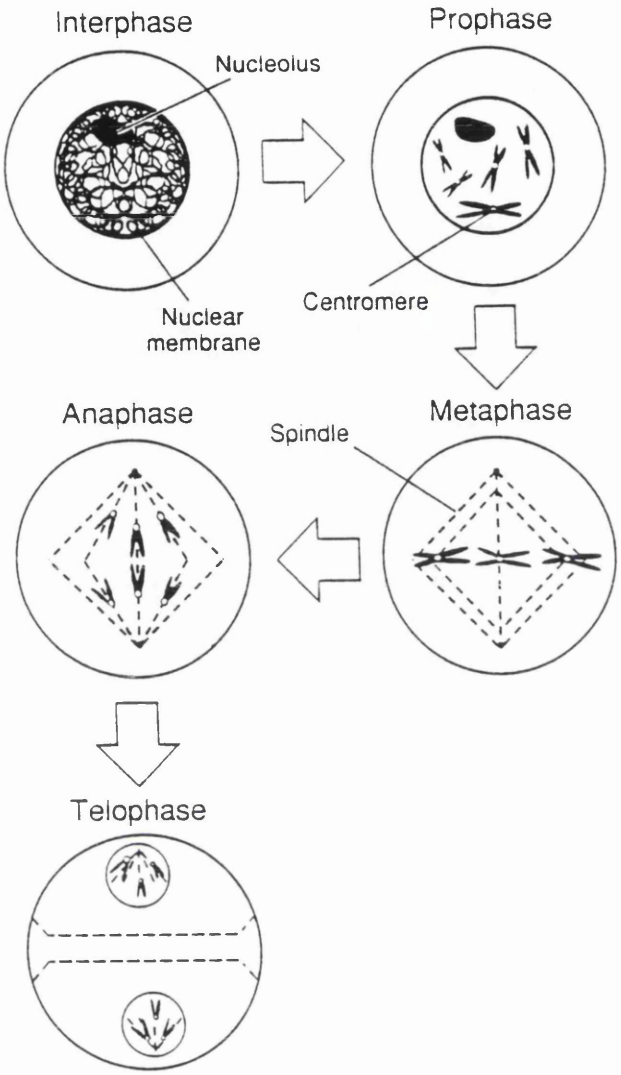


Fig 1.4 The stage of mitosis (adopted from Mueller and Young, Emery's Elements of Medical Genetics, 1995)

iv) Anaphase

Anaphase begins with the division of the centromeres and consequently starts to separate the chromatids. The spindle fibers drag the sister chromosomes to opposite ends of the cell.

v) Telophase.

The final stage of mitosis is telophase. In telophase, the chromosomes uncoil, become less distinct, and the individual chromosomes become indiscernible again. The nucleolus and the nuclear membrane reappears, the spindle fibers disappear. During or immediately after telophase, the cytoplasm is divided by the formation of new cell membranes in a process called cytokinesis, and finally the cell division is completed. The mitosis results in two daughter cells, each with a complete and identical set of genetic information.

1.4.2 The Cell Cycle (Normal)

The cell cycle consists of a series of two major events which result in DNA duplication and cell division. The eukaryotic cell cycle consists of the M (mitotic) period and interphase. Interphase is further divided into G_1 (Gap 1), S (Synthetic), and G_2 (Gap 2) phases (Fig 1.5).

An interphase cell is not in the dividing state but is active in metabolism. DNA replication takes place during S-phase. Although the length of the cell cycle varies considerably from one species and tissue to another, the average mammalian cell cycle takes 18 hours, with nine hours for G_1, five hours for S, three hours for G_2, and one hour for M.

G_1 phase is the period for preparing to synthesize DNA and for synthesis of both RNA and proteins. Although DNA replication occurs during S phase, some DNA will replicate early, and other DNA will replicate late. A given part of the chromosome will almost always replicate at a certain time in the S period. During G_2 phase, cells are preparing for mitosis. At the beginning of G_1 the cell may start into a new cycle immediately, or enter a non-cycling stage known as G_0. In G_0 phase, the cell remains in a transient quiescent state until stimulated

to return to the cell cycle or terminally differentiated state (Lawce and Barch, 1991; Bradley et al., 1995; Carr, 1996)).

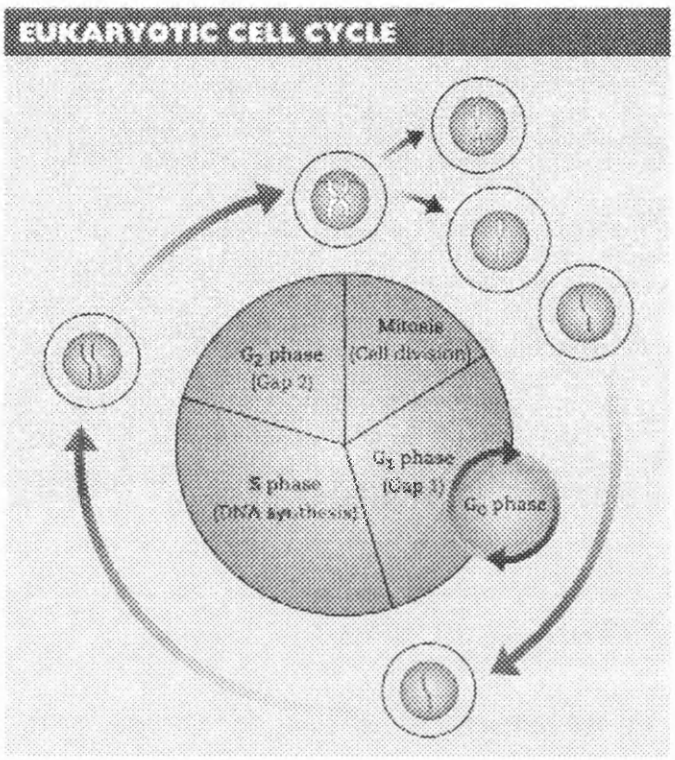


Fig 1-5 Cell cycles (Adopted from Bradley et al.; Lecture notes on molecular medicine, 1995)

1.5 Progression of Human Cytogenetics

1.5.1 Brief history of development in human cytogenetics

One of the oldest parts of genetics is cytogenetics which is a study of the chromosome itself.

The first important development in human cytogenetics was the discovery of the

exact number of the diploid human chromosomes in somatic cells as forty-six by Tjio and Levan in 1956. This discovery using cultured embryonic lung cells, made use of the earlier discovery (Hsu, 1952) that hypotonic treatment improved the spreading of metaphase chromosomes. In the same year, Ford and Hamerton (1956) studied meiotic chromosomes in spermatocytes and observed 23 bivalents.

The major technical development of the use of phytohemaggutinin (PHA) to stimulate the peripheral blood T lymphocytes to divide (Moorhead et al., 1960) provided a more accessible source of suitable metaphases available for cytogenetic analysis. Colchicine was first used in cytogenetic studies during the 1950s (Clark and Wall, 1996).

In 1959 the first chromosome aberrations in man were described; the presence of an additional chromosome 21 in patients with Down Syndrome was shown by Lejeune et al. (Lejeune et al., 1959). Turner syndrome was associated with a 45,X chromosome constitution (Ford et al., 1959) and the 47,XXY karyotype of Klinefelter's syndrome was demonstrated by Jacobs and Strong (Jacobs and Strong, 1959).

Initially chromosomes were uniformly stained by Feulgen or Orcein and attempts at identification were based on overall chromosome size and arm ratio. The development of the first banding technique, Q banding (Casperson et al., 1968) allowed identification of individual chromosome pairs and chromosome regions. G-banding described by Seabright (1970) allowed chromosome identification without the use of fluoresence. Since then more than a dozen chromosome banding methods have been introduced (Verma and Babu, 1995).

These developments led to the description of many more chromosome abnormalities, and to the detailed genotype/phenotype correlations which are the basis of diagnostic cytogenetics.

1.5.2 General Banding techniques

Since the development of fluorescent banding by Caspersson's group, many banding methods have been developed, such as G-banding, R-banding, Q-

banding.

G-banding is the most popular banding method in the diagnostic cytogenetics laboratory. Chromosomes treated with methanol-acetic acid fixation are morphologically best for this method. G-banding of chromosomes is a two–stage process including pretreatment using trypsin and staining with Giemsa or Leishman's stain or Wright stain. Enzymatic digestion using trypsin is damaging to the protein content but not to the DNA of the chromosomes. Chromosomes are divided into light and dark bands by G-banding. G-light bands were shown to replicate in the first half of S phase followed by replication of G-dark bands in the last half of S phase (Holmquist et al., 1982).

In diagnostic cytogenetics, approximately 400 to 2000 G-bands can be produced on each haploid chromosome set. Chromosomes are divided into three types depending on the position of the centromere: metacentric, acrocentric, and submetacentric. By standard nomenclature of the human karyotype, p is for the short arm of a chromosome, q for the long arm of a chromosome. Bands are numbered outwards from the centromere, using an agreed classification of regions and bands.

R-banding, reverse-banding, gives essentially the same information as G-banding and Q-banding. R-banding is particularly useful for the study of structural changes involving chromosome ends that might be difficult to detect by G-banding due to normally light staining with G-banding in those regions. One interesting finding about R-dark (G-light) bands is that they mainly contain the housekeeping genes of the cell and about half of the tissue–specific genes, are rich in GC and have CpG islands. On the other hand, dark staining regions were found to be 3.2% richer in A and T than G-light bands (Holmquist et al., 1982) and have relatively few active genes, implying a structural compartmentalization of the genome (Benn and Perle, 1992; Clark and Wall, 1996).

C-banding reveals regions of constitutive heterochromatin, which are situated at the centromeres of all chromosomes, at the pericentromeric regions of chromosome 1, 3, 9, 16 and at the distal end of the long arm of the Y. The

distribution of C-bands is very species–specific or even individual–specific (Clark and Wall, 1996). C-banding can be a useful method for detecting whether such variants are due to heterochromatin or are of clinically significant euchromatin.

Q-banding with certain fluorescent dyes gives a banding pattern similar to G-banding with Q bright bands corresponding to G dark bands, and Q dull bands to G light bands.

There are also a number of banding techniques staining a specific area of the chromosome such as RE staining, in situ hybridization banding, D-banding, and AgNOR banding.

1.5.3 Types of chromosomal aberrations

Chromosome aberrations are chromosomal changes caused by mutation, which are large enough to be visible under the light microscope and are divided into two categories: numerical abnormalities and structural abnormalities. Both of them may involve either the autosomes or the sex chromosomes.

Numerical aberrations (polyploidy, aneuploidy)

Aneuploidy is usually caused by the failure of paired chromosomes or sister chromatids to disjoin at anaphase (non-disjunction) or delayed movement of a chromosome at anaphase (anaphase lag). Aneuploidy can occur during meiosis or mitosis, and meiotic non-disjunction may occur at the first or the second meiotic divisions.

Triploidy is usually caused by fertilization with two sperm (dispermy) or by failure of one of the maturation divisions of the egg or the sperm.

Tetraploidy usually occurs due to failure to complete the first zygotic division.

Structural aberrations

Structural aberrations occur due to chromosome breakage and then the unstable ends of the chromosomes induce translocation, deletion and ring chromosomes, duplications, inversions, isochromosomes, and centric fragments (Fig 1.6).

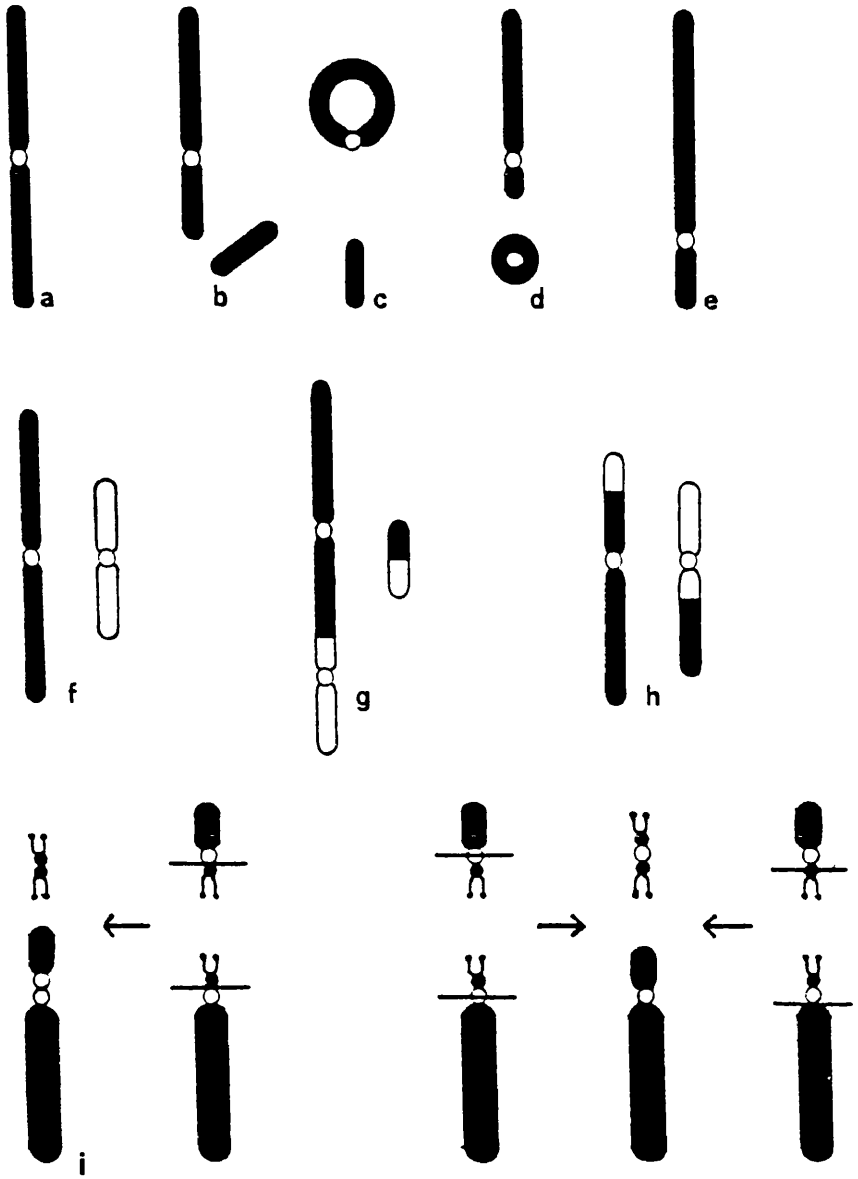


Fig 1.6 Diagram of structural aberrations (adopted from Therman and Susman, Human chromosomes, 1993)

(a) breaks in one chromosome, (b) broken chromosome; (c) centric ring and acentric fragment; (d) acentric ring and centric fragment; (e) chromosome with pericentric inversion; (f) breaks in two chromosomes; (g) dicentric chromosome and acentric fragment; (h) balanced reciplocal translocation; (i) Robertsonian translocations

i) Translocation

The exchange of chromosomal material between chromosomes is caused by breakage of both chromosomes with repair in an abnormal arrangement, or accidental recombination between non-homologous chromosomes during meiosis. There are three types of translocation - reciprocal, centric fusion (Robertsonian), and insertional. Where no chromosomal material is missing translocations may have no phenotypic effect on the individual carrying them but have consequences in the risk of abnormal offspring.

ii) Deletions

A deletion is a chromosomal mutation involving the loss of a segment of a chromosome. The missing region of chromosome may be interstitial or terminal. The consequence of the deletion depends on the genes or part of the genes which have been lost.

iii) Ring chromosome

It is caused by breakage of long and short arms of the same chromosome with rejoining and loss of material beyond the breakpoints. Depending on the amount lost, it may have phenotypic consequences. Topological problems in replication may be caused by different alignments of rejoining.

iv) Isochromosome

After misdivision or breakage of the centromere, sister chromatid rejoining immediately adjacent to the centromere leads to production of a chromosome with formation of two long arms or two short arms of the same chromosome.

v) Inversion

In inversion, part of a chromosome has been rotated through 180°. Inversions are not necessarily harmful, but may cause problems in the pairing of homologous chromosomes in meiosis. Where the centromere is involved it is called a pericentric inversion; where both breakpoints are in the same arm this is called a paracenric inversion.

vi) Duplication

This, an additional copy of a segment of a chromosome, may result from unequal crossing over during meiosis and meiotic events in a parent with a translocation, inversion or isochromosome.

1.5.4 Limitation of chromosome banding technique

Chromosome banding techniques have been able to identify specific human chromosomes and chromosomal aberrations. However they have some limitations as a diagnostic or research tool. Chromosome banding techniques need high quality metaphase spreads from actively dividing cells but these are sometimes very difficult or impossible to prepare from certain cell types or tissues, especially hematological malignancies and solid tumours. Although particular chromosomal bands can be recognized by their position in a chromosome, small chromosomal aberrations less than a few megabases of DNA, such as microdeletions, and inversions, do not always have sufficient characteristic bands for precise identification.

Conventional cytogenetic techniques also sometimes have difficulty in identifying the composition of abnormal chromosomes present in complex karyotypes such as derivative chromosomes or marker chromosomes.

The interpretation of chromosome banding patterns is often technically difficult, especially with respect to detection of minor structural changes and when analyzing complex karyotypes, such as those of highly aneuploid tumor cells (Lichter et al., 1988).

1.6 Fluorescence In Situ Hybridisation

The basic principle of in situ hybridisation is that when a DNA probe is applied to a cytological preparation under the optimal conditions, it will find and hybridise to its complementary sequences in the cell or chromosome. It was

devised and first used as a method for gene mapping by Pardue and Gall (1969) and independently by John et al. (1969). Initially it was limited to highly repeated probes until the advent of recombinant technology enabled the cloning and purification of any DNA sequence. At first, visualization was by means of incorporation of radioisotopes, but this method had several disadvantages such as long exposure time, short shelf life because of half-life of isotopes, decreased banding resolution, inconvenience and lack of safety (Dyer and Meyne, 1991). Because of these disadvantages ISH moved to other methods for recognition of gene localization of which the most popular is the use of fluorochromes. These can either be incorporated directly into the probe DNA (FITC or rhodamine etc.) or detected indirectly via use of biotin or digoxigenin.

The development of FISH allowed diagnostic applications to evolve to supplement classical cytogenetic banding techniques leading to the development of molecular cytogenetics. It is particularly useful in detecting the origin of small derivative chromosomes and demonstrating the presence of deletions beyond the limit of resolution of the light microscope. This method can not only study metaphase chromosomes but also interphase cells and paraffin-embedded sections. FISH in interphase is mainly useful in detection of aneuploidy of specific chromosomes but may also demonstrate the presence of some specific translocations, mainly in malignancies.

1.6.1 Basic Principles of FISH

The FISH technique is based on the double-stranded nature of the target and probe DNAs, which are rendered single stranded DNA after denaturation. The probe DNA will anneal with their complementary sequences in metaphase preparation or interphase nuclei under suitable conditions. A general outline of the in situ hybridisation procedure is presented in Fig 1.7.

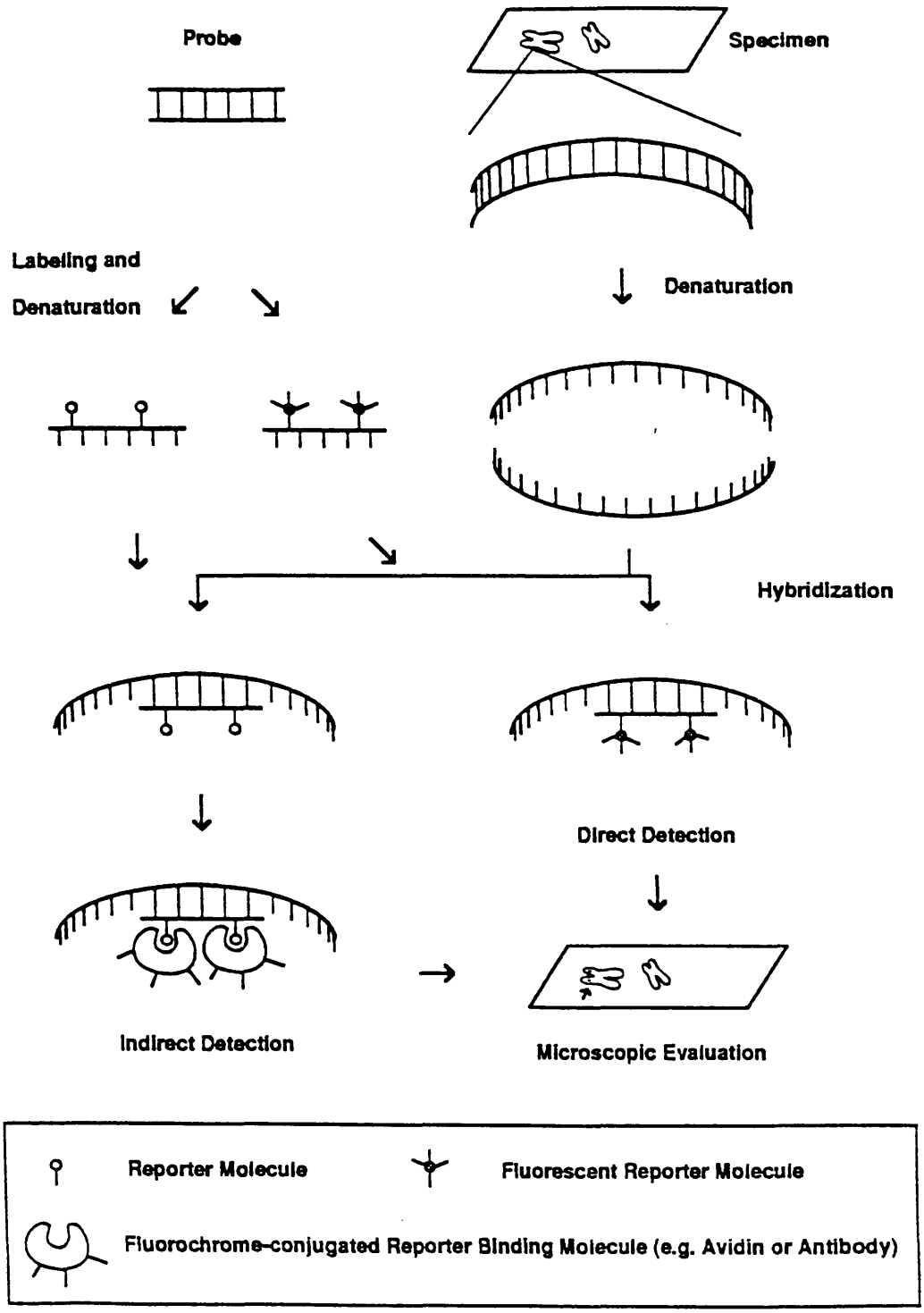


Fig 1.7 Schematic illustration of FISH (adopted from Lichter and Cremer, Chromosome analysis by non-isotopic in situ hybridisation; In Human cytogenetics A practical approach vol.1, 1992)

Target

The first requirement for successful FISH is target material of high quality to maximize probe hybridisation efficiency and to decrease the signal-to-noise ratio. A chromosome preparation should contain minimal cytoplasm and other cellular debris because these reduce the signal intensity and produce high levels of background. The chromosomes should also be well spread to allow accurate analysis.

Probes

DNA sequences in target DNA are usually detected using labelled DNA probes. These probes are usually cloned sequences in vectors such as plasmids, cosmids or YACs, and are normally double–stranded. Synthetic oligonucleotides are made using a DNA synthesizer and PCR also is able to amplify DNA sequences (up to 4 kbp in length) as probes.

Labelling

i) Labels

A wide variety of non-radioactive labels have been used in recent years. There are two main types of labelling.

a) Direct labelling- In this approach a detectable molecule (reporter) has been incorporated into the probe and can be visualized directly after hybridisation to the target nucleic acid. Some fluorochromes (e.g. FITC, rhodamine) can be detected directly.

b) Indirect labelling- the reporter molecules are incorporated into the probes using nick translation, then after hybridisation, they are detected by binding with fluorescent affinity reagents. The two most frequently used reporter molecules are biotin and digoxigenin. Biotin is vitamin H found in egg white, usually

incorporated in form of biotin-11-dUTP, and detected via either anti-biotin antibody or biotin-streptavidin system for which it has a strong affinity. Digoxigenin is a steroid found in *Digitalis purpura* and *D. Lanata,* incorporated as digoxigenin-11-dUTP, and is detected via antibodies.

ii) Labelling Method;

a) Enzymatic labelling by nick translation (Fig 1.8)

Biotin, digoxigenin and fluorochromes can be incorporated into probe DNA using enzymatic labelling systems of which the most popular is nick translation. This reaction uses two enzymes, DNase 1 and DNA polymerase 1. The DNase 1 produces single strand nicks in the double stranded DNA of the probe to expose a free 3′-OH group. The 5′ to 3′ exonuclease activity of DNA polymerase 1 deletes mononucleotides at the 5′ side of the nick to synthesize a copy of the template strand. The function of polymerization of DNA polymerase catalyses the incorporation of new dNTPs from solution at the 3′OH end of nick. The ideal size of probe for maximizing specific hybridisation and decreasing background signals is about 200-400 bp (Trask, 1991). This can be achieved by adjusting incubation time or ratio of enzyme mixture (DNase : DNA polymerase 1).

b) Chemical labelling

AAF (Acetylaminofluorene), sulfone groups, and mercury can be chemically introduced into double stranded and single stranded DNA, and RNA and detected by immunochemistry.

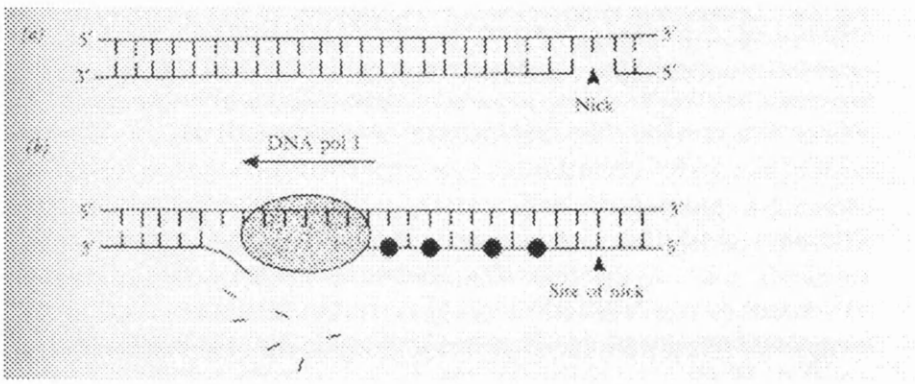


Fig 1.8 Diagram of nick translation (Adopted from Nicholl; An Introduction to Genetic engeneering, 1994).

Hybridisation:

i) Conditions for the denaturation

The labelled DNA is then hybridised to metaphase chromosomes or interphase nuclei after the denaturation of both the DNA of the target cells and the labelled probe DNA. Double stranded DNA of both target and probe sequences need to be denatured to single stranded DNA by dissociation of hydrogen bond between two complementary nucleic acids strands. There are several factors affecting the stability of the hybrid nucleic acid -the proportion of guanine and cytosine, the length of hybrid nucleic acid, the environment of the nucleic acid hybrids, and the presence of mismatched hybrids. The degree of denaturation for the target DNA is more critical than that for the probe because of the narrow window between adequate denaturation and DNA loss.

ii) Conditions for the reannealing

Hybridisation depends on the ability of denatured DNA to reanneal with complementary sequences. For binding to complementary sequences in the target, the hybridised slides are incubated overnight at 37°C, which is usually about 20-25°C below the melting point. The Tm is the temperature at which half the DNA is present in a single stranded form. The Tm value is different for

genomic DNA isolated from various organisms. The rate of hybridization depends on the probe length, complexity of sequence and concentration.

Post-hybridisation washing

Non-specifically bound probes can be dissociated by washing at various stringencies. The stringency of the washes can be adjusted by changing the formamide concentration, salt concentration and temperature.
Post hybridisation washes are usually carried out in 50% formamide (v/v)/2x SSC and 2x SSC at 42-45°C to remove weakly bound probe leaving absolutely perfectly or mostly perfectly matched nucleotides in the duplex.

Detection of the probe

To produce a fluorescent signal at the site of probe hybridisation, different haptens, including biotin and digoxigenin, can be detected with immunochemicals linked to different fluorochromes. In the indirect method, slides are treated with immunofluorescent reagents such as fluorochrome-conjugated avidin or anti-biotin antibodies for biotinylated probes. Detection of hybridised digoxigenin probes is mediated by high affinity anti-digoxigenin antibodies conjugated to alkaline phosphatase, peroxidase, fluorescein or rhodamine (Fig 1.9). When probes are too small to make strong signals properly, several cycles of amplification can be applied (Fig 1.9).

Visualization of the signals

After mounting in an anti-fade solution containing DAPI, the slide is evaluated using a fluorescence microscope equipped with an UV mercury lamp and appropriate excitation and emission filters.

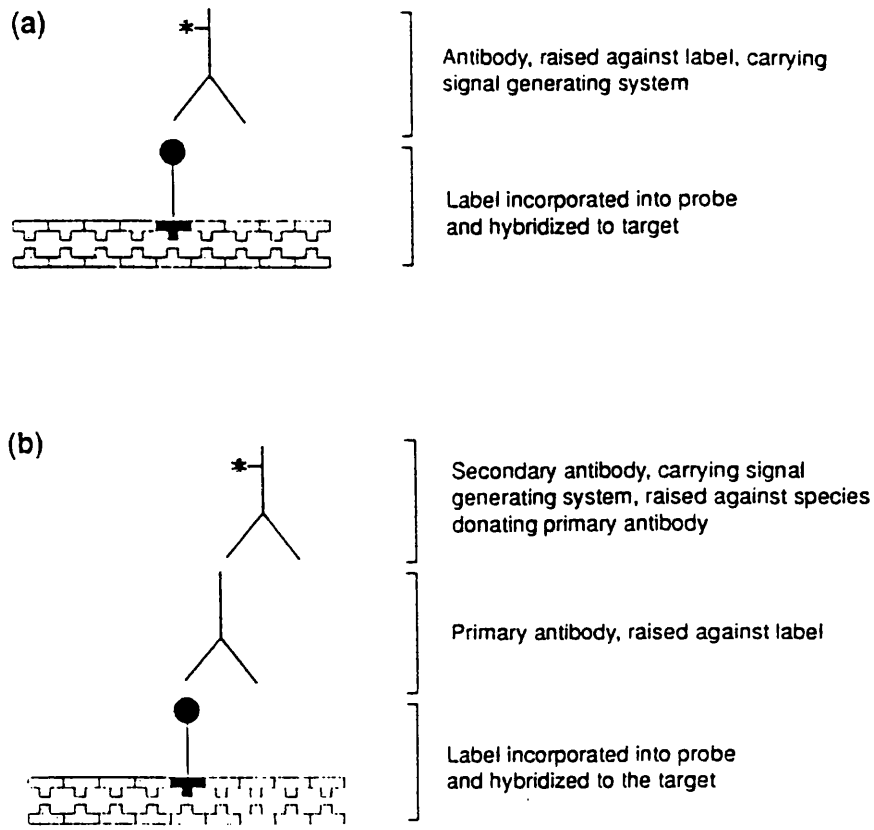


Fig 1.9 Immunochemical detection and signal-generating system for non-radioactive probes. a) One step detection system b) Two-detection system. (adopted from Leich et al., In situ hybridization, 1994)

1.6.2 Types of probes and their applications

Probes for FISH include repetitive element probes, whole chromosome paint probes, single-copy probes, and species-specific total genomic DNA probes.

Whole chromosome libraries (paints)

Whole chromosome paint probes are derived from flow-sorted chromosomes or from somatic cell hybrids that contain only a single human chromosome. They are cloned in lambda phage vectors (Van Dilla et al., 1986) or in pBS plasmid vectors (Fusco et al., 1989; Collins et al., 1991) or by the PCR amplification of flow-sorted chromosomes.

These probes are composed of sequences representing loci along the length of an entire chromosome. Because these probes usually contain interspersed repetitive sequences such as the Alu and Kpnl elements chromosomal in situ suppression hybridisation is needed to suppress cross hybridisation with common repetitive sequences by using appropriate competitor DNAs. The suppression hybridisation technique utilizes a partial reannealing step to block binding by the non-specific repetitive DNA present in all human chromosomes. The labelled probe fragments and a competitor DNA are denatured together and then incubated at 37°C for a given period of time (usually up to an hour) to allow preannealing (Lichter and Cremer, 1992).

Whole chromosome paints are useful for detection of chromosome rearrangements such as translocations, duplications, and possibly marker chromosomes in some constitutional chromosome abnormalities, the acquired chromosomal changes of leukemia and other malignancies (Leversa, 1993). A limitation for this probe is the difficulty of detecting abnormalities in cryptic regions, and also structural changes within the same chromosome (Yung, 1996). Detection of small deletions and small chromosomal rearrangements is also difficult.

Chromosome-specific repetitive element probe

These probes usually consist of highly repetitive sequences, approximately present in 100 to 5000 copies in specific chromosomal regions such as near centromeres, telomeres, or heterochromatic regions on specific chromosomes. There are thus various kinds of probes with repetitive sequences such as alpha-satellite centromeric probes and telomeric probes (Trask, 1991).

The most informative chromosome-specific repetitive element probes are chromosome specific centromeric probes, composed of alphoid DNA repeats. These probes are widely used to identify numerical abnormalities in metaphase spreads or interphase nuclei in prenatal diagnosis, and cancer genetics (Lichter, 1991).

Single-copy probes (chromosome specific unique sequence probes)

These probes show a variable range of size depending on the vector (plasmid→cosmid→yeast). Single copy probes can be used to detect complementary unique sequences in metaphase and interphase cells. These probes have great advantages for identification of structural abnormalities such as micro-deletions (eg. Williams syndrome; Yung, 1996), inversions such as inv(16) found in AML (Trask, 1991; Dauwerse et al., 1993)), and translocations (eg. *bcr/abl*; Tkachuk et al., 1991; Bentz et al, 1994a). The hybridisation efficacy of these probes depends on the size of probe applied. As probe size becomes larger, the hybridisation efficacy increases, ranging from 20-50% with plasmid probes containing 2 kbp of target sequences to more than 90% using large-insert probes such as cosmid or YACs (Yeast Artificial Chromosomes) (Trask, 1991).

Species-specific total genomic DNA probes

Total genomic DNA (consisting of the entire DNA complement of an organism's genome) can be used as a probe to identify individual chromosomes in cell fusion hybrids.

Discrimination between two genomes is greatly improved when the genomic probe is hybridised in the presence of an excess concentration of unlabelled total genomic DNA from the other genome (blocking DNA) as CISS.

Interphase FISH

Fluorescent in situ hybridisation is especially useful for the detection of chromosomal aneuploidy and the investigation of chromosomal organization in interphase nuclei. FISH on metaphase spreads is sometimes limited by a lack of sufficient mitoses for analysis. Interphase FISH is independent of metaphase cells by hybridising directly to non-dividing cells. Thus there is no requirement for lengthy cell culture and this is particularly important in malignancies where chromosomal changes not present in the original tumour might arise.

FISH to nuclei is particularly useful in situations where the number or quality of metaphase chromosome preparations is inadequate for full analysis as can be the case in some types of leukemia and is enormously useful in assessing the success of bone marrow transplantation. It is also valuable where results are required quickly (e.g. prenatal diagnosis of common aneuploidies) or where prolonged culture time might lead to abnormalities arising in vitro.

In prenatal diagnosis, centromeric probes are used to screen for chromosome X and 18 in uncultured amniocytes or fetal cells isolated from maternal blood (Leversa, 1993). Unfortunately there is cross hybridisation between the centromeric probe for 13 and 21, thus the 13/21 shows 4 signals in normal cells and five in cells with aneuploidy making scoring difficult. In these cases, complex probes such as genomic fragments (without clustered repeats) cloned in cosmids, Pl, YACs, or similar vectors are applied. Good results are obtained with chromosome specific plasmid clones (Lichter et al., 1988b) or YACs for chromosome 21 (Chumakov et al., 1992).

Probes specific for alpha satellite DNA can also be used to look for aneuploidy of specific chromosomes in malignancies where they allow many cells to be screened, for examples, hyperdiploidy in ALL (Moorman et al, 1996), trisomy 12 in CLL (Escudier et al., 1993). In solid tumours, aneuploidy can be detected

by tissue in situ hybridisation.

Single copy probes may also be used in interphase cells to look for specific abnormalities eg. *bcr/abl* for the Philadelphia chromosome (Bentz et al, 1994a), t(15;17) (Warrel et al., 1993), inv(16) in AML (Trask et al., 1991; Gray et al., 1992; Dauwerse et al., 1993) and deletion of the human erythrocyte ankyrin gene on chromosome 8 associated hereditary spherocytosis (Lux et al., 1990).

Chromosome specific libraries are unsuitable for detection of aneuploidy in interphase cells. It has been shown that each chromosome occupies as specific domain in the interphase nucleus and consequently the signal obtained from FISH using chromosome libraries is diffuse, overlapping and confusing to interpret (Tkachuk et al., 1991).

1.6.3 Additional applications of FISH

Reverse chromosome painting

The principle of this procedure is that labelled DNA from various sources is hybridised to metaphase chromosome spreads from normal cells by in situ suppression hybridisation. This approach can be applied to investigate the chromosomal content of interspecies hybrid cells, to identify the origin of marker chromosomes (Carter et al., 1992) and to recognize unbalanced chromosomal material of entire genomes by using the whole genomic DNA as a probe (Joos et al., 1993).

Micro-FISH

Micro-FISH is a composite procedure involving microdissection of an aberrant chromosomal region, amplification by degenerate oligonucleotide primed-PCR and labelling of the dissected DNA, and subsequent FISH to normal metaphase chromosome (reverse chromosome painting).

Micro-FISH can be used for the analysis of chromosomal aberrations, especially in malignant cells and de novo mutations, which often remain incompletely

resolved because of the limitations of routine banding and staining techniques in the identification of small and complex chromosome rearrangements.

The micro-FISH technique has also been called reverse chromosome painting, originally described by Carter et al. (1992) as opposed to forward chromosome painting. With this method, cytogenetically unknown origin of the marker chromosomes, balanced or unbalanced translocations, duplications, interstitial deletions, and insertions can be directly identified even without any clue from the conventional cytogenetic method (Meltzer et al.,1992; Xu et al., 1995; Muller-Navia et al., 1996). Although this method is very sensitive and straightforward, it still requires reasonable banded patient metaphases for chromosome microdissection and is difficult to optimize because it involves several techniques, all of which are critical.

Fibre-FISH

This visual high-resolution genome mapping technique is based on FISH of probes to free chromatin fibres derived from lysed cells (Heiskanen et al., 1995). To achieve better resolution, FISH is performed on extended single DNA fibres. There are several methods for extending single DNA fibres, which are collectively referred to as Fibre FISH (Florijn et al., 1995). These methods have been employed in ordering of overlapping and adjacent probes in the range 1 - 100kb (Parra and Windle, 1993; Senger et al., 1994). Fibre-FISH may also be very useful for high resolution physical mapping, and contig evaluation, and for detecting genetic aberrations, such as microdeletions, translocations, inversions, and small duplications (Wiegant et al., 1992; Parra and Windle, 1993), with an accuracy far superior to that possible by traditional FISH techniques.

Multicolour-Primed in situ labelling

The Primed In Situ Labelling (PRINS) technique is an alternative to traditional FISH methods for detection of nucleic acids in situ. Multi PRINS is a fast and simple method by which allows simultaneous probing of multiple repetitive

sequences. The basic principle of PRINS is that an unlabelled oligonuceotide probe is used as a primer for chain elongation in situ, using labelled deoxynucleotides as substrate for a DNA polymerase. The target chromosomal DNA acts as a template for the chain elongation. Newly synthesized DNA can be identified by fluorescence detection (Hindkjær et al., 1994,1996).

In comparison with FISH, PRINS is a very fast procedure; less than 1 hour is required with repetitive DNA sequences and less than 3 hours with unique DNA sequences. Gosden et al., (1991) showed high quality chromosome R-banding could be produced by PRINS with Alu oligonucleotides as primers. Koch et al, (1991) applied direct incorporation of fluoresein-12-dUTP to the PRINS to avoid the time consuming immunocytochemical steps to detect the labelled probe.

PRINS can be used to simultaneously visualize multiple sequences, both in metaphase and interphase chromosomes. Multi- PRINS is a valuable tool to identify unambiguous chromosomal aberrations and aneuploidies from a small number of suitable metaphases.

Chromosomal bar codes

After many attempts to devise a more specific and flexible way of chromosome banding, the term bar was developed to distinguish such a chromosome subregion more clearly than the natural chromosome bands. Any combination of bars can be applied to mark chromosomes at multiple, selected sites and consequently produce a chromosomal bar code (CBC). For applications of CBCs as a general diagnostic tool, large number of probes and improved hybridisation efficiency would be required (Lengauer et al., 1993).

Although multi-colour CBCs are definitely not enough to screen whole chromosomes for unknown aberrations, a specific CBC can provide comprehensive information in a single FISH experiment for identification of the suspected translocations, insertions, deletions, and inversions by a change in a sequence of differently coloured bars spaced along the chromosomes of interest (Lengauer et al., 1993).

1.6.4 Approaches to Whole Genomic Screening

Multicolour FISH

Although FISH can identify some chromosome abnormalities with a high degree of specificity, it is usually used with some prior knowledge or suspicion of the abnormality likely to be present, and usually not more than two probes with different fluorochromes are applied simultaneously. One approach to obtaining a global analysis using FISH was developed to multicolour FISH. At the beginning, Nederlof et al. (1989b) developed a three-colour FISH for the simultaneous detection of multiple nucleic acid sequences using three fluorochromes.

Multicolour FISH was developed to identify each of the 22 human autosomes and the two sex chromosomes with distinctively different colours. It is possible to obtain a unique spectral colouring on each chromosome by using a hybridising set of chromosome specific DNA probes labelled with a different combination of fluorescent dyes. Two strategies enable detection of targets beyond the number of fluorochromes; one is called combinatorial labelling, where each target is identified by probe labelled with a defined combination of fluorochromes: the other is called ratio labelling, where each target is recognized by a combination of fluorochromes at different ratio (Reid et al.,1992a,b; Le Beau, 1996). In multicolour FISH, multiplex FISH (M-FISH) and spectral karyotyping (SKY) are two major approaches (Speicher and Ward, 1996). M-FISH depends on digital images obtained separately for each of the five fluorochromes (FITC, Cy3, Cy3.5, Cy5, Cy7) using a CCD camera. Then special software system analyses these by generating a composite image in which each chromosome is pseudo-coloured based on its fluorochrome composition. In the SKY system, an interferometer is used to analyse the spectrum of fluorescence wavelength for each pixel of the CCD chip. The special computer software identifies the components of this spectrum by applying a classification algorithm. These two methods have advantages and also disadvantages. Applications of M-FISH and SKY have not yet been established, but these methods provide

comprehensive screening of whole karyotypes for detecting chromosomal aberrations in a single FISH experiment. Multicolour FISH is particularly valuable for identification of the origin of marker chromosome, especially in solid tumours and for the detection of balanced chromosomal aberrations. Proper metaphase preparations are an essential part of multicolour FISH, but they are difficult to obtain from many tumour samples especially hematologic malignancies. Decreasing mitotic cells or displacement with another clone during cell culture are another problem of metaphase preparations. Multicolour FISH showed limitations in the detection of paracentric inversions, some pericentric inversions, insertions involving a single chromosome arm, small duplications and small deletions. Also, the sensitivity in detecting cryptic telomeric translocations was not established. Now, attempts are being made to apply multicolour FISH to interphase nuclei for visualization of all chromosome domains simultaneously (Speicher and Ward; 1996).

1.6.5 Microscopy

Fluorescence Microscopy and filters

The basis of fluorescence microscopy lies in the fact that a photon of a particular wave- length (excitation wavelength) excites an electron in the fluorochrome and the excited electrons become unstable, and on returning to a stable state lose energy which is emitted as light (Leitch et al., 1994). Although the average wavelength of emitted fluorescence is longer than the average excitation wavelength for any given fluorochrome, there is in fact some overlap for many commonly used fluorochromes.

To generate and observe flurorescence, it requires a fluorochrome bound to a molecule, a light source, and a detection system with appropriate optical filter for the particular fluorochrome to produce and to detect various excitation and emission wavelengths.

Excitation filters are used to select the correct wavelengths of the light for the specific fluorochrome and other wavelengths are either absorbed or reflected by

this filter. The emission or barrier filter is also required for separating unabsorbed excitation light from the fluorescence emission light because most of the excitation light is not absorbed by the fluorochrome. Filter sets vary according to the amount of light which they permit to pass such as long band-pass filters, narrow band-pass filters, and dual- and triple band-pass filters enable to simultaneously visualize two or three fluorochromes.

Light sources

The light source for fluorescence microscopy is a mercury lamp. Mercury lamps can be chosen by desirable excitation intensities that correspond to bulb wattage (50W or 100W). Brighter excitation intensity produces more intense fluorescence and therefore a shorter exposure time for photographs. A 100 W bulb also has a longer operating life than a 50 W bulb. Mercury bulbs should not used >200 hr because of a risk of explosion that can damage the microscope.

1.7 Comparative Genomic Hybridisation (CGH)

CGH, which was first developed by Kallioniemi et al in 1992, is a comprehensive method for detection of DNA sequence copy number changes (deletions, gains, and amplifications) on a genome-wide scale in a single hybridisation using extracted test DNA as a probe.

Unlike FISH, CGH analysis does not need previous information about cytogenetic aberrations, nor does it require a series of probes to fully evaluate the extent of genetic gains or losses in a test sample. Conventional cytogenetics is hardly used in the analysis of solid tumours because of technical difficulties in culture. CGH was developed as a method of overcoming this limitation since it allows analysis of tumour DNA without requiring chromosome preparations from malignant tissue.

1.7.1 Basic principles of CGH

CGH is based on competitive binding of test and control DNA to normal metaphase chromosomes. The DNAs are differentially labelled with different fluorochromes (green and red) and co-hybridised to a normal metaphase under suppression conditions (Fig 1.10). The basic assumption in CGH is that the hybridisation kinetics of the test and control DNA are independent, so the ratio of the binding of the DNA to a specific locus is proportional to the ratio of the copy numbers of the sequences in the two DNAs. The relative intensities of both differently labelled genomic DNAs (test and control) to normal metaphase chromosomes reveals the region of changed copy number. Ratios of the signals can be quantified along the length of each homologue and provide a copy number karyotype of the test DNA (Kallioniemi et al., 1992; du Manoir et al., 1993).

Cohybridisation of differentially labelled normal DNA (control DNA) provides an internal standard, which allows differentiation between variation in signal intensities due to differences in DNA copy number and variations due to signal inhomogeneities. This is caused by several experimental parameters which include differential denaturation of chromosomal regions with different base composition, and chromatin packaging as well as different target accessibility of probe sequences (Bentz et al., 1994b).

At present CGH is applied to detect genetic aberrations in a wide spectrum of cancers. The use of the CGH technique in cytogenetic analysis of solid tumours has expanded rapidly in particular where archival formalin fixed paraffin-embedded sections are used as starting material. Paraffin block studies are especially useful in many tumour types in which fresh tissue is usually not available, for example, skin melanoma (Isola et al., 1994).

Paraffin block CGH provides a powerful research tool to study the pathogenesis of cancer by evaluating genetic progression from pre-malignant to fully malignant lesions, or by comparing genetic aberrations in primary tumours and their metastases.

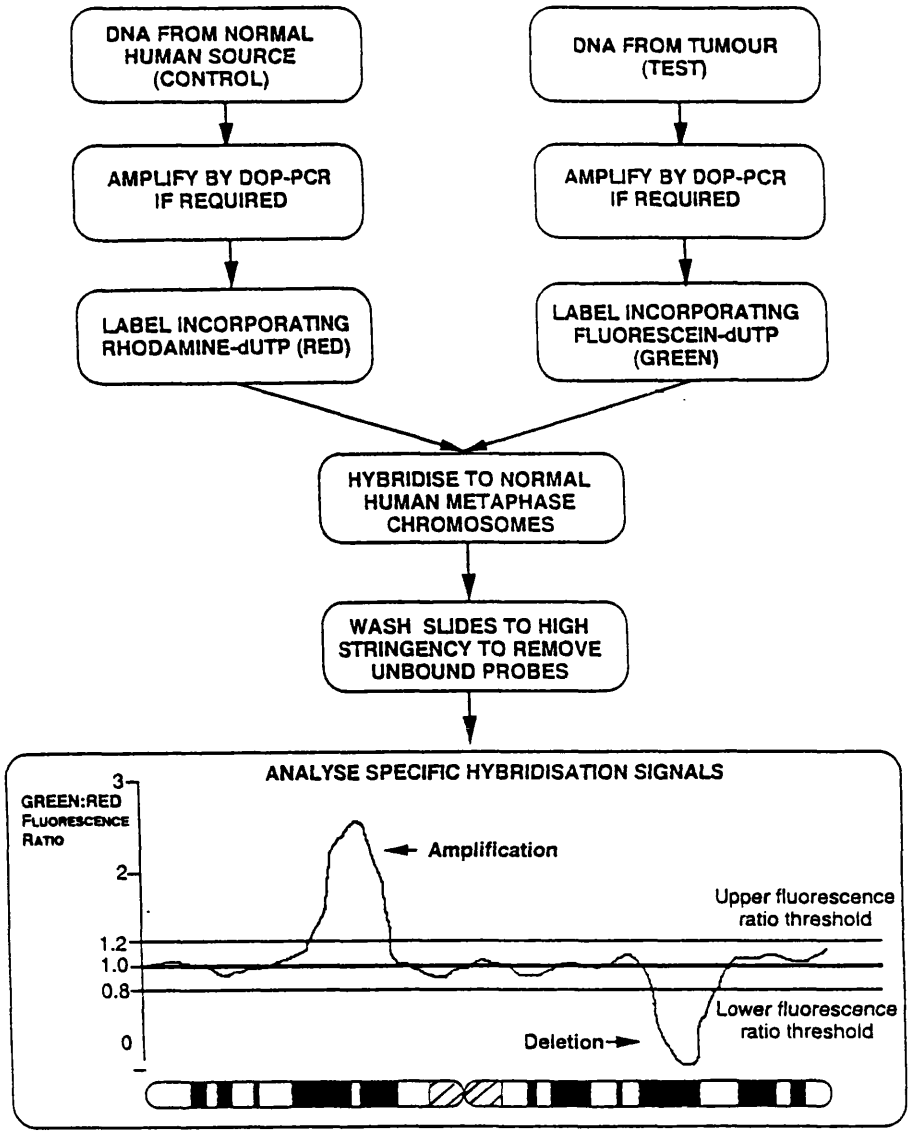


Fig 1.10 Schematic illustration of CGH (adopted from James et al., Journal of pathology 187: 386, 1999)

1.7.2 Preparation for CGH experiments

Normal metaphase preparations for CGH

Normal metaphase chromosomes prepared from phytohaemaggulutinin (PHA)-stimulated, synchronised peripheral blood lymphocytes from a karyotypically normal healthy donor are used as hybridisation targets in CGH.

The quality of metaphase preparations is crucial to obtaining an optimal CGH image. It is desirable to prepare several batches of slides at once before selecting a suitable one. The hybridisation characteristics of several slides from each batch of slides are tested using labelled DNAs from a control cell line. When high quality hybridisation cannot be achieved, the entire batch has to be abandoned. The best quality of metaphase preparation contains reasonably straight chromosomes with minimal overlapping, adequate length of chromosomes and a high mitotic index with little cytoplasmic residue.

Isolation of DNA for CGH

The CGH analysis of disease processes requires the extraction of test DNA from a sample of blood, bone marrow, or a variety of tissue preparations including fresh and paraffin embedded tissue.

The technique for the extraction of DNA from cytologic preparations and fresh tissue is already well established. Fixation and paraffin embedding of tissue is a procedure that has a profound effect on its molecular arrangement. The DNA of frozen tissue sections is still present as high molecular weight DNA and good quality CGH can be achieved even after DOP-PCR with direct, standard cell lysis protocols. In contrast, the formalin fixation of tumour tissues leads to extensive cross-linking of nuclear proteins and formation of tight complexes between proteins and DNA, in addition to fragmentation of DNA; therefore formalin fixation is the major limiting factor for the DNA analysis of archival material (Isola et al., 1994). The success of a CGH study using fixed, paraffin

embedded materials depends on several factors, including the fixative used in the tissue processing, the duration of the fixation, the age of the paraffin block, and the length of the DNA fragment to be amplified. Several technical modifications to improve the yield of high molecular weight DNA from paraffin embedded tissues have been suggested, such as modifying proteinase K application, changing the extraction buffer, or incorporating an additional incubation step with sodium thiocyanate after the dewaxing (Speicher et al., 1993). From a literature review, it appears that prolonged proteinase K treatment can improve the DNA extraction efficacy in relatively thick tissue sections. Although the underlying mechanism remains unknown, this effect may be related to partial reversal of fixation induced cross-links in aqueous buffer during proteinase K digestion (Isola et al., 1994). In very small volumes of microdissected samples, single-step cell lysis method may be used for DNA extraction because the number of cells is too small to extract by the standard method (Zhuang et al., 1995). Another problem of DNA extraction is contamination of malignant cells with normal cells from the surrounding tissue and with necrotic cells, resulting in decreased specificity of CGH results.

Probe labelling

Probe labelling for CGH is carried out by nick translation in the same way as for FISH. In CGH experiments with very small amounts of test DNA, DOP-PCR labelling is also performed during the amplification process.

Nick translation

The length of the probe molecules after labelling is a crucial factor for good quality CGH. In CGH it is important to adjust not only the optimal length of the fragments but also to produce a similar length (quality) for test and reference DNA.

During the nick translation process, probe size can be modified by adjusting the

ratio of DNase 1 to DNA polymerase 1 in the reaction solution and/or the incubation time. The optimum size for ideal CGH is between 500 to 2000 bp (Kallioniemi et al., 1994). As with FISH, labelling may be by direct or indirect methods. In the indirect method, biotin and digoxigenin are used commonly as reporter molecules owing to their sensitivity and commercial availability. In direct labelling, directly fluorochrome-conjugated nucleotides, such as FITC-11-dUTP and TexasRed-5-dUTP are used.

Denaturation, Hybridisation and Washing

Basic theory for denaturation and hybridization is the same as in FISH. For DNA:DNA in situ hybridisation both the probe and target sequences need to be denatured to make them single stranded prior to in situ hybridisation. In CGH the normal metaphase slides as target DNA are denatured at 72-74°C for 3 minutes in 70% formamide/2x SSC (Kallioniemi et al., 1994), but optimal denaturation time and temperature varies from one batch of slides to another. After denaturation, slides should be checked for chromosome morphology to obtain the optimal denaturation time.

In some papers, proteinase K treatment after denaturation is recommended (du Manoir et al., 1993; Kallioniemi et al., 1994). Although pretreatment of the specimen by proteolytic digestion can improve the probe accessibility to specimen, the effect is controversial because such a treatment may also result in a higher granularity of the signal by the genomic DNA.

Probes, which are mixtures of equal amounts (120-200 ng) of test and control DNA with Cot-1 DNA (5-10 ug), are also denatured at 70°C for 5 minutes (Kallioniemi et al., 1994).

Hybridisation essentially follows the steps used in chromosome suppression in situ hybridisation with unlabelled Cot-1 DNA to block binding of the labelled repetitive sequences in both genomes. For accurate assessment of chromosomal imbalances by variations in the fluorescence intensity ratio, suppression must be as complete as possible.

Hybridisation is carried out under a coverslip in a moist chamber at 37°C to 39°C for 2-3 days. The temperature and duration of incubation can be changed depending on test DNA conditions.

Post-hybridisation washing is carried out as standard FISH protocols. When indirectly labelled probes are used, washing procedure is performed as in standard dual colour FISH protocol with immunochemical staining with avidin-FITC and anti-digoxigenin rhodamine (Kallioniemi et al., 1992, du Manoir et al., 1993).

Image acquisition and Analysis of CGH

For proper CGH analysis, there are two basic requirements; one is hardware for image acquisition and the other is software for image analysis. For hardware, a conventional epifluorescence microscope with 50W or 100W mercury lamp, appropriate excitation and emission filter sets, and camera are employed. Although a cooled CCD (Charge Coupled Device) is not needed, it has many advantages including digital readout, high spatial resolution, high sensitivity, low noise, and high dynamic range (du Manoir et al., 1995b).

Software for image analysis of CGH is developing continuously. Image analysis can be achieved by two methods. One is global analysis which involves examining individual metaphases at the level of individual picture elements (pixels) calculating the fluorescence ratio and coding to various colours according to the magnitude. The other is axial analysis which is a method of calculating the average red:green ratio along a defined line, calculating the average intensity value for a specific pixel width at right angles (orthogonal) to the line, generating a graph and painting the abnormal regions. Finally the fluorescence ratios over six metaphases are collected and represented in a CGH Karyogram.

For proper CGH analysis, several steps of analysing procedure are performed in addition to assessment of image quality. These are background correction; segmentation to separate the chromosomes; identification of the chromosomes

by the banding pattern of the DAPI image; normalisation of the fluorescence intensities from the test and reference DNA; measurement of the ratio of test and reference signals along lines perpendicular to the chromosome axis; averaging of fluorescence ratio from multiple chromosomes; and interpretation of the ratio profiles.

i) Assessment of image quality

For reliable CGH analysis, six or more metaphase spreads are selected in each experiment. Every metaphase should be assessed by objective criteria including degree of suppression of repeat sequence in the hybridisation, intensity of the hybridisation signals, signal to background ratio, homogeneity of the hybridisation, homogeneity of the image, and granularity of the hybridisation to increase the quality of CGH images (du Manoir et al., 1995b)

ii) Correcting the background

Local background fluorescence and residual uneven illumination can be corrected by subtracting the interchromosomal fluorescence intensity from the chromosomal fluorescence.

iii) Segmentation of images

Chromosome segmentation is for defining accurate chromosomal boundaries by pre-filtering in the image through a top-hat filter, or by basing segmentation on the combined DAPI and reference DNA image or possibly by using a different counterstain with better telomere staining properties. Segmented images are created by setting zero value for pixels that are outside the chromosome masks, eventually it will be used to retain all material within the green area.

iv) Identification of chromosomes

Specific chromosomes could be identified visually based on the R-banding pattern in the DAPI image. Actually DAPI banding does not show a very distinct banding pattern to allow definite identification. Identification could be facilitated using grey scale DAPI component by enhancing the banding pattern and

displaying using an inverted lookup table.

v) Normalisation of the fluorescence intensity (Fluorescence equalisation)

To compare information among different copies of the same chromosome type in different metaphases and in different experiments, some sort of normalisation or standardisation procedure is required. This procedure produces approximately equalised median intensities of the test and reference images in all metaphases. Thus the corrected median test/median reference ratio is approximately equal to 1.0 for each metaphase and fluorescence ratio of the chromosomal area corresponding to the normal part of the test DNA will become approximately 1.0 in all images.

vi) Determining of ratio profile

Test and reference fluorescence intensity profiles are obtained by combining the pixel value along slices orthogonal to the chromosome axis. The chromosome axis is calculated using a standard skeleton operator. Point by point division of the intensity profiles yields the ratio profile.

v) Profile averaging

Averaging of ratio profiles to reduce random noise and give better estimation of copy number differences is required. A minimum of 5-10 examples of each chromosome is needed to obtain average ratio profiles. Chromosome length standardisation is required before averaging ratio profiles. Then, average ratio profiles are calculated by combining the individual ratio profiles from multiple images and displaying the averaged ratio profiles together with a measure of variability on CGH Karyogram (du Manoir et al., 1995a,b).

vi) Identifying regions of copy number gains or loss

Determination of threshold is also an important step in detecting DNA copy number changes accurately. CGH analysis procedures result in measurements of relative copy number only. Most interpretations are based on whether the mean profile at a locus, or the mean ratio value for an entire chromosome arm, is either

higher than an upper threshold, or less than a lower threshold. Upper thresholds of between 1.10 and 1.25 and lower thresholds between 0.75 and 0.90 are currently used. Particularly the wider threshold (0.75-1.25) is the theoretical threshold for monosomy and trisomy in cases where a chromosomal imbalance is present in 50% of cells (du Manoir et al, 1995b). However it may be insufficiently sensitive in cases where a chromosome imbalance is present in only a small proportion of the cells contributing the test DNA. Alternative thresholds using statistical distribution of the average ratio value for balanced chromosomal regions in tumour sample also can be applied. Confidence intervals (95% or 99%) express the error estimated, which is directly proportional to the standard error. By using these statistical justifications, sensitivity for detection of small regions of DNA copy number change may be increased (du Manoir et al., 1995b).

Limitation and difficulties of CGH

Generally CGH is limited by resolution of affected chromosome regions. In a digital image system, deletion is less detectable than gain of copy numbers.
CGH can only detect genetic aberrations that involve true losses or gains of DNA sequences. Thus balanced translocations or inversions, point mutations and small intragenic rearrangements cannot be detected. Also CGH can only detect relative DNA sequence copy number changes, so that diploid cells cannot be differentiated from true triploid or tetraploid cases by using the ordinary method of analysis. Peri-centromeric and heterochromatic repeat regions, notably the centromeric region of 1, 9, 16, and Y and acrocentric short arms, cannot be reliably evaluated by CGH as they are blocked to various extents by the unlabelled Cot-1 DNA in the hybridisation and there are no unique sequence hybridisation signal in these area. The ratio changes at or near these regions should be analysed carefully because these DNA sequences are highly polymorphic in copy number between individuals. Telomeric regions should also be carefully investigated because of the fall off in the hybridisation signal at the

very end of chromosomes. Some regions of chromosomes, for example GC rich regions such as 1p32-pter, 16p, 19 and 22, occasionally show widely variable intensity ratios and this is thought to be related to the structure of those chromosomes. Another frequent problem for CGH is caused by mosaicism involving two different cell lines, or in the case of tumours with contamination of normal cells. If the normal cell contamination is greater than 50% of the total DNA content, reliability of CGH is decreased. In bi- or multi clonal tumours, it is difficult to detect the different genetic aberrations present in the individual clones because CGH detects the average copy number of sequences in all cells included in the specimen.

1.8 Development of the CGH Technique using Whole Genome Amplification

1.8.1 Polymerase Chain Reaction (PCR)

Specificity and Fidelity of PCR

PCR is designed to selectively amplify specific target DNA sequence or sequences within a heterogeneous collection of DNA sequences.
The efficacy of PCR can be measured by its specificity, yield, and fidelity. Although an ideal PCR aims to get a product with high specificity, yield, and fidelity, adjusting conditions for maximum specificity may not be compatible with high yield. Likewise, optimising for the fidelity of PCR may result in reduced efficiency. The buffer conditions, the PCR cycling regimen (i.e., temperature and duration of each step), and DNA polymerase would affect the success of PCR.

i) Template
Suitable substrates for PCR include all kinds of DNA and RNA such as genomic,

plasmid, and phage DNA, previously amplified DNA, cDNA, and mRNA. In general, the efficiency of PCR is greater for smaller-size template DNA than for high-molecular-weight (undigested eukaryotic genomic) DNA. PCR can be used to amplify as little as a single molecule of template, although great care has to be taken when performing such experiments, particularly in the avoidance of contamination.

ii) Primer design

An ideal set of primers should hybridise efficiently to the target sequence with minimum hybridisation to other related sequences that are present in the sample. Primers are generally 15-30 nucleotides in length with an approximately equal number of each of the four bases. Primer pairs should also be designed so that there is no complementarity of the two bases at extreme 3′ ends either inter or intra individual primers to reduce the incidence of primer-dimer formation (Newton and Graham, 1994). The specificity of amplification depends on the specificity of primer hybridisation to the target site with respect to different target DNA sequences, which are not intended to be amplified. Primers which take part in nonspecific extension in PCR not only reduce the concentration of primer available for the correct amplicon but can also lead to artifactual products, or false positive or negative results.

iii) Reaction Mixture

The standard buffer for PCR with Taq polymerase contains 50mM KCl, 10 mM Tris-HCl (pH 8.3 at room temperature), and 1.5 mM $MgCl_2$. Although the standard buffer works well for a wide range of templates and oligonucleotide primers, the buffer for a particular PCR should be optimised depending on the target, the primer sequences, and the concentration of other components in the reaction such as dNTP and primers. Optimisation of the concentration of Mg^{++} is very important whenever a new combination of target and primers is first used or when the concentration of dNTPs or primers is changed. dNTPs are a major source of phosphate groups in the reaction, and any change in their concentration affects the concentration of available Mg^{++} (Cha and Thilly, 1995).

iv) Primer and dNTP

The ratio between the primer and template is an important factor, which affects the specificity of PCR. If the ratio is too high, PCR is more prone to generate nonspecific amplification products, and primer-dimers are also produced. However, if the ratio is too low (<0.1% of the standard condition for the genomic DNA PCR), the efficiency of PCR is greatly decreased. Unincorporated primers are completely dependent on the amount of target sequences generated, on the other hand unincorporated dNTPs depends not only on the number of target sequences generated, but also on the size of the target sequence. Thus, for amplifying a large target sequence, a higher concentration of dNTP is recommended (Keohavong et al., 1988).

v) PCR Cycle

A standard PCR cycle consists of three steps: (1) a denaturation step (1-2-minute incubation at > 94°C); (2) a primer annealing or hybridization step (1-2-minute incubation at 50°-55°C); and (3) an extension step (1-2-minute incubation at 72°C).

Although higher annealing temperature and shorter time of extension step improve the specificity of PCR, large fragments (>1 kb) of template DNA are needed to increase the duration of each step to get efficient amplification (Kwok et al., 1990).

vi) Exponential phase of PCR

An understanding of the kinetics of specific product accumulation during PCR is necessary for an informative and analytical PCR. The desired blunt-ended duplex fragments start from the third cycle of the PCR, and then this product accumulates exponentially according to the formula, $N_f = N_0 (1+Y)^n$, where N_f is the final copy number of the double-stranded target sequence, N_0 is the initial copy number, Y is the efficiency of primer extension per cycle, and n is the number of PCR cycles under conditions of exponential amplification (Keohavong et al.; 1988). In most cases, after the final copy number of the

desired fragment (N_f) reaches about 10^{12}, its efficiency per cycle (Y) drops dramatically due to limitation of enzyme action. Continuing PCR beyond this point often produces amplification of nonspecific bands, the appearance of small deletion mutant bands, and the disappearance of the specific product in certain instances (Cha and Thilly, 1995). Because each specific PCR amplification has a different efficiency, an accurate quantitative analysis should be carried out to determine the efficiency of the particular PCR.

Method to reduce PCR Contamination

PCR is especially susceptible to contamination because PCR produces an abundance of amplified DNA product from a small amount of DNA. There are three sources of contamination such as DNA from other test samples, DNA from experimental materials such as recombinant clones, or DNA generated by previous PCR amplification of the same target sequences (carryover contamination).

To avoid contamination during PCR, experiments should be set up in a laminar flow cabinet or in a separate laboratory. Equipment and reagents for PCR should be kept separately from general laboratory equipment. Reagents which can be autoclaved should be sterilized in an autoclave. Disposable gloves and lab coat should be worn at all times.

i) Ultraviolet irradiation to reduce PCR contamination

UV irradiation of dry DNA or DNA in solution acts as a tool to prevent PCR contamination. Most UV-induced DNA damage occurs via the formation of cyclobutane rings between neighboring pyridine bases, thymidine or cytidine because the cyclobutane rings produce intrastrand pyrimidine dimers that inhibit polymerase-mediated chain elongation (Cone and Fairfax, 1995). Three-dimensional objects, such as pipettes, cannot be effectively decontaminated by UV light because only a fraction of the surface actually exposes the light source. Although UV irradiation provides an additional margin of safety for keeping the PCR laboratory contamination-free, meticulous technique remains the most

important method for preventing contamination. In particular, UV irradiation cannot be substituted for the physical separation of sample preparation in pre- and post PCR experiment.

Generally, UV irradiation for more than 10 min with 100μJ min^{-1} at 254 nm in a UV crosslinker should be recommended for reaction tubes and the reaction mixture (except the sample DNA, primer and DNA polymerase) (Newton and Graham, 1994).

Various kinds of Enzyme for PCR amplification

i) DNA polymerase 1 from E.coli

This heat labile enzyme was the first polymerase to be identified and has the function of polymerization of DNA in a 5′→3′ direction, 5′→3′exonuclease activity for nick translation , 3′→5′ exonuclease activity for proofreading.

ii) Taq/AmpliTaq DNA polymerase

The thermostable DNA polymerase from *Thermus aquaticus* has been the most widely used enzyme in PCR. Taq DNA polymerase has an optimal extension rate of 35-100 nucleotides per second at 70°-80°C which is the optimum temperature range for the enzyme. Taq DNA polymerase has a 5′ to 3′ exonuclease activity, which removes nucleotides ahead of the growing chain. But this enzyme has no 3′→5′ exonuclease activity to perform a proofreading function. The fidelity of Taq (2×10^{-4} error/bp per duplication) is the lowest among DNA polymerases whose fidelity has been measured.

Since AmpliTaq is recombinant, the purity and reproducibility of this enzyme are higher than Taq DNA polymerase. AmpliTaq LD is a recombinant, thermostable 94kDa DNA polymerase encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene which has been inserted into an *Escherichia coli* host. This enzyme is identical to AmpliTaq DNA polymerase but is further purified through a proprietary process to reduce bacterial DNA introduced from the environment. This product is especially useful for PCR

amplifications of low copy number bacterial target sequences (<1000 bp) because of the low level of DNA in the preparations.

A modified version of the recombinant AmpliTaq DNA polymerase (Stoffel fragment) is also available which has a deletion of 289 amino acids from the N terminus. This version has twofold higher thermostability, no intrinsic 5′ to 3′ exonucease activity compared to the Taq or Amplitaq DNA polymerases, exhibits optimal activity over a broader range of magnesium concentrations.

iii) Topoisomerase 1

Topoisomerase 1 catalyzes unwinding superhelical structures from covalently closed circular double-stranded DNA by transient breakage and rejoining of phosphodiester bonds (Sambrook et al., 1989).

iv) T7 Sequenase

T7 Sequenase (version 2.0 DNA polymerase) is derived from bacteriophage T7 DNA polymerase but chemically or genetically modified to improve its properties for sequencing. These properties include heat-labile, high processivity, low 3′ to 5′ exonuclease activity. T7 DNA sequenase acts well at the low temperature at which the random primer complexes become stable and it has strand displacement synthesis capabilities which can synthesize long stretches of DNA by displacing other primers that have already annealed to the DNA (Bohlander, 1992; Guan et al., 1993).

v) Pfu DNA polymerase

This polymerase is isolated from the hyperthermophilic marine archaebacterium *Pyrococcus furiosus* and has both 5′ to 3′ DNA polymerase activity and 3′ to 5′ exonuclease proofreading activity. The 3′ to 5′ exonuclease activity enhances the fidelity of DNA synthesis as it will excise incorrectly added, mismatched 3 terminal nucleotides from the primer/ template and then incorporate the correct nucleotide. The fidelity of DNA synthesis is 12-fold higher than that of Taq DNA polymerase (Newton and Graham, 1994).

vi) Pwo DNA polymerase

Pwo DNA polymerase is isolated from *Pyrococcus woesei* and possesses 3′ to 5′ exonuclease activity but no 5′ to 3′ exonuclease. This enzyme also has proofreading activity as Pfu DNA polymerase. This enzyme has a half life of 2hr at 100° C and is useful for high temperature primer extension reactions, DNA cycle sequencing and blunt-ending cloning (Newton and Graham, 1994).

1.8.2 Whole Genome Amplification (WGA) from a very small amounts of DNA

Molecular or molecular cytogenetic analysis of very small amounts of DNA is limited because of insufficient material to perform more than one independent PCR amplification or CGH. To solve this problem, there are several methods to generate a non-specific amplification of all genomic sequences (whole genome amplification) such as primer extension preamplification, degenerate oligonucleotide primed PCR, alu-PCR, tagged PCR and ligation-mediated PCR. Successful whole genome amplification requires that the fidelity of the sequences be maintained and that the extent of amplification is not significantly biased. Degenerate oligonucleotides are used as primers in PCR to induce polymerase extension randomly at multiple sites in total genomic DNA template to reach these requirements.

The reliability of whole genome amplification, particularly in a single or a few microdissected formalin-fixed and paraffin-embedded solid tumour cells, has not yet been systematically evaluated. Also the limiting cell number of microdissected tissue sections has likewise not yet been systematically investigated for CGH experiment. There are many problems in achieving properly amplified samples. First, when tissue sections are microdissected and used for DNA analysis by PCR (WGA), the diminished volume of the cell and nucleus by cutting may represent loss of chromosomes or part of chromosomes, probably leading to false negative CGH results (Speicher et al., 1995). Another problem is intrinsic error rate of Taq polymerase (error rate: 10^{-4}/bp), which is

prone to introduce AT-to-GC transitions as well as to generate deletion mutations due to secondary structures of the DNA template (Cha and Thilly, 1995). Proof-reading polymerases (e.g. Pfu polymerase) have not yet been fully evaluated for the sequence accuracy. Thus there is a potential risk of getting incorrectly amplified sequences during whole genome amplification when concentration of template DNA is extremely low.

Primer Extension Preamlification (PEP)

basic primer:
15-base oligonucleotides (NNNNNNNNNNNNNNN)
The primer is composed of a mixture of 4^{15} (1×10^9) sequences.

Primer extension preamplification (PEP) is an in vitro procedure developed to amplify a large fraction of the genome from limited amounts of DNA, such as that derived from a single cell and from microdissected paraffin-embedded tissue sections.

PEP was first developed by Zhang et al. (1992). PEP involves repeated primer extension using a mixture of 15-base random oligonucleotides and the Taq DNA polymerase. It is one of the universal genomic amplification methods which have been developed to overcome limitations of PCR analysis in very small amount of DNA. Several groups have used PEP, with minor modification such as different concentration and size of primer, changing enzyme or changing cycles, especially in preimplantation genetic diagnosis (Xu et al., 1993; Kristjansson et al., 1994; Snabes et al., 1994). It is estimated that PEP can cover at least 78% of the genomic sequence in a single human haploid cell and can amplify any sequence in the genome to a minimum of 30 copies. Because of the random nature of the primers, it is also unlikely that any one primer-extension product will be involved in primer extension at a subsequent cycle more than any other primer extension product (Zhang et al., 1992).

When PEP was applied to a single diploid cell or very small cell free DNA samples, careful consideration had to be given to the size of each aliquot and the

number of aliquots taken from the PEP reaction because of the missing allele in the aliquot (Zhang et al., 1992). In the case of paraffin-embedded samples, whole genome amplification using PEP was not easy because of inadequate amount and the poor quality of template DNA which was cross-linked with proteins and already nicked to relatively short fragments. Although PEP amplification of the whole genome, coupled with subsequent locus-specific amplification by PCR, represents a significant improvement over PCR alone for the genetic analysis of a single cell, the application of PEP for CGH was still under investigation.

Degenerate Oligonucleotide-Primed PCR (DOP-PCR)

Basic primer;
5′-CCG ACT CGA GNN NNN NAT GTG G-3′

DOP-PCR was designed by H. Telenius, 1992 as a simple PCR technique to produce general amplification of target DNAs by multiple locus priming without restriction using a partially degenerate sequence as a primer. This degeneracy, combined with a PCR protocol with a low initial annealing temperature, leads to multiple priming at evenly dispersed sites within a given genome. Furthermore, the method appears to be species-independent because efficient amplification is achieved from the genomes of all species tested using the same method. Thus, for the general amplification of target DNA, DOP-PCR proved more effective than interspersed repetitive sequence PCR (IRS-PCR), which relies on the appropriate position of species-specific repeat elements (Telenius et al., 1992).
During the DOP-PCR, several cycles of low temperature annealing and extensions can anneal at many sites in the human genome. During the first few cycles, the annealing temperature usually does not exceed 30°C, since this facilitates annealing of the short sequences specified by the 3′ end of the oligonucleotides used, hence allowing sufficient priming to initiate DNA synthesis at frequent intervals along the template. Six random bases can yield 4^6

primers of different sequences as opposed to the single sequence of a non-degenerate primer, thus making its applicability universal. After several cycles of low temperature annealing and extensions, the annealing temperature is increased to bind more specific priming sites on 6-bp 3′ end and specific 10-bp 5′ end. It will allow specific hybridisation to the tagged-genomic sequences only, and exponential increase in DNA will take place until Taq DNA polymerase is exhausted. While it is possible that higher amounts of Taq DNA polymerase may improve the overall yield, this can produce an increased amount of primer related products.

Although DOP-PCR amplified exponentially with lower starting DNAs, genomic DNA below certain level produces an increased chance that a given locus will not be represented in the DOP-PCR product.

DOP-PCR can be used to achieve several hundred-fold amplification of virtually all sequences in the human genome with sufficient starting template genomic DNA. The product amplified by DOP-PCR cannot represent the whole genomic sequence of the starting genomic DNA and universally DOP-PCR amplified one-third as well (Cheung and Nelson, 1996). This is thought be caused by non-specific priming and consequently creating non-specific DNA, which has no relation to the human genome and by overlapped sets of DNA fragments. Additionally, over-represented highly repetitive DNAs may be related (Cheung and Nelson, 1996).

The CGH technique in association with DOP-PCR allowed large retrospective prognostic studies of genetic aberrations as well as studies on genetic progression from premalignant lesions to advanced lesions in solid tumours by application of the CGH technique to the DNA obtained from very small amounts of paraffin embedded tissue samples.

1.8.3 Probe Labelling

DOP-PCR labelling

Degenerate oligonucleotide primer PCR may also be useful for labelling genomic DNA. This method is another version of PCR labelling by employing a heat stable DNA polymerase. The cycle of denaturation, primer annealing, DNA synthesis is repeated up to 40 times. The heat stable DNA polymerase has been shown to accept modified nucleotides (digoxigenin or biotin labelled nucleotides, directly fluorochrome-conjugated nucleotides) as substrates. The PCR can be used not only to amplify DNA but also to produce large quantities of labelled probe DNA suitable for CGH.

Purification of labelled probe

After labelling procedure, final product in the mixture includes labelled probe and unincorporated labelled nucleotides. These unincorporated nucleotides are better to be removed to avoid non-specific background signals.
The High Pure PCR Product Purification Kit (Boehringer Mannnheim) is made for the efficient and convenient isolation of PCR products without primer, mineral oil, salts, unincorporated nucleotides and the thermostable polymerase from amplification products. The High Pure PCR Product Purification Kit (Boehringer Mannnheim) is based on the principle that nucleic acids bind specifically to the surface of glass fibers or silica materials in the presence of a chaotropic salt. The binding reaction occurs within seconds by the disruption of the organized structure of water molecules and the interaction with the nucleic acids. Since the binding process is specific for nucleic acids, the bound material can then be separated and purified from impurities (e.g. salts, free nucleotides and proteins) by a simple washing step. The small oligonucleotides and dimerized primer from PCR products are also selectively removed by synergistic process for requiring minimum lengths of the DNA fragment.

Rapid Labelling Assay

One of the critical problems associated with non-radioactive labelling is the question of how much labelled probe has been produced because it is difficult to monitor incorporation of labelled nucleotide. In this rapid assay, the labelled probe is dotted onto a membrane (Hybond-N + nylon or whatman DE 81), the unincorporated nucleotide is eliminated in a 20 minutes wash at 60°C. The remaining bound probe is visualized by a UV transilluminator or by eye in the dark room and compared with diluted original fluorochrome as known standards (100%, 75%, 50%, 25%, 12.5%, 6.25%).

1.8.4 Validation of the CGH results

There is a potential risk of false copy number changes being introduced during whole genome amplification by methods such as PEP or DOP-PCR. Many efforts have been made to validate CGH with other cytogenetic methods. Comparison of CGH results with those obtained by karyotyping, FISH, and molecular method (e.g. microsatellite assay or loss of heterozygosity) has clearly indicated that CGH reliably detects DNA sequence copy number aberrations of solid tumour (Kallioniemi et al.; 1994).

To obtain reliable detection of chromosome gains and losses, careful controlling of the quality of hybridisation is required. This can be evaluated by the signal strength, the smoothness of the signal distribution along the chromosome, the lack of accentuated banding, the efficient blocking of the centromere, and the absence of artifactual ratio variations. One of the important ways of verifying the CGH results is to include normal and positive controls in each experiment.

Inverse labelling CGH and tissue in situ hybridisation are also used for validation (Isola et al., 1994; Speicher et al., 1995).

Inverse labelling CGH (Reverse CGH)

To further improve the accuracy of the CGH, inverse-labelling can be applied. Inverse labelling means that test samples are labelled with Texas-Red and hybridised with FITC-labelled normal reference DNA to control for differences in hybridisation of FITC versus Texas-Red labelled probes. Comparison between these two hybridisation methods confirmed the presence of copy number aberrations at almost all loci (Isola et al., 1994; Speicher et al., 1995).

Tissue In Situ Hybridisation

FISH has become a well-established technique to confirm CGH results. Particularly in solid tumors where fresh cells or freshly prepared fixed cell preparations were not available, paraffin-embedded tissue can become an alternative source of material for FISH (Schofield et al., 1992; Kim et al., 1993). Paraffin-embedded tissue can be analysed as a suspension of disaggregated, but intact, nuclei or as an intact section of tissue. The advantages of performing studies on suspensions of intact nuclei are that numerical abnormalities for a given chromosome can be detected even in a small population of cells. The main disadvantage is loss of the spatial relationships of one cell to another. The main advantage of analysing tissue sections is preservation of the spatial relationship between cells and possibility of identification of numerical abnormalities in subpopulation of cells. The major drawback to this method is that exact counting of abnormal cells is very difficult because of the three dimensional structure of the cells and the existence of partial nuclei rather than intact nuclei in a given 4 to 6-um-thick tissue section. To increase the accuracy of the test, many nuclei must be counted and statistically analysed to determine whether an abnormal population of cells exists.

1.9 Genetic Changes in Cancer Development

Cancer is caused by disruption of the genetic mechanisms that control the normal cellular growth, location and mortality of cells. In most of cancer, the genetic mutations are not inherited and arise in somatic cells during adulthood as a result of exposure to environmental carcinogens (Connor and Ferguson-Smith, 1993) or of spontaneous genetic changes but some individuals have an inherited mutation in a gene which induces a genetic predisposition for development of cancer (Hodgson and Maher, 1993). Mutation in three broad categories of genes including proto-oncogenes, tumour suppressor genes, and genes coding for DNA repair enzymes probably leads to disruption of normal control mechanism by changes in cell surface protein expression, protein secretion and cell motility (Fig 1.11). Cancer development is associated with an inter-relation between susceptibility to cancers and an impaired ability of cells to repair DNA damage. It suggests that cancers develop not only from deregulated proliferation but also from disorganized control of the programmed cell death or apoptosis. Growth arrest process associated with aging (senescence) may become useless as a further consequence of the activation of oncogenes or the loss of tumour suppressor genes. Senescence is usually associated with shortening the length of telomeres which are composed of short, tandem repeats of the hexanucleotide 5′-TTAGGG-3′ at the end of each chromosome. Telomerase, which is responsible for synthesis of the telomere, is active in many tumours but is not common in normal tissue (Hesketh, 1995).

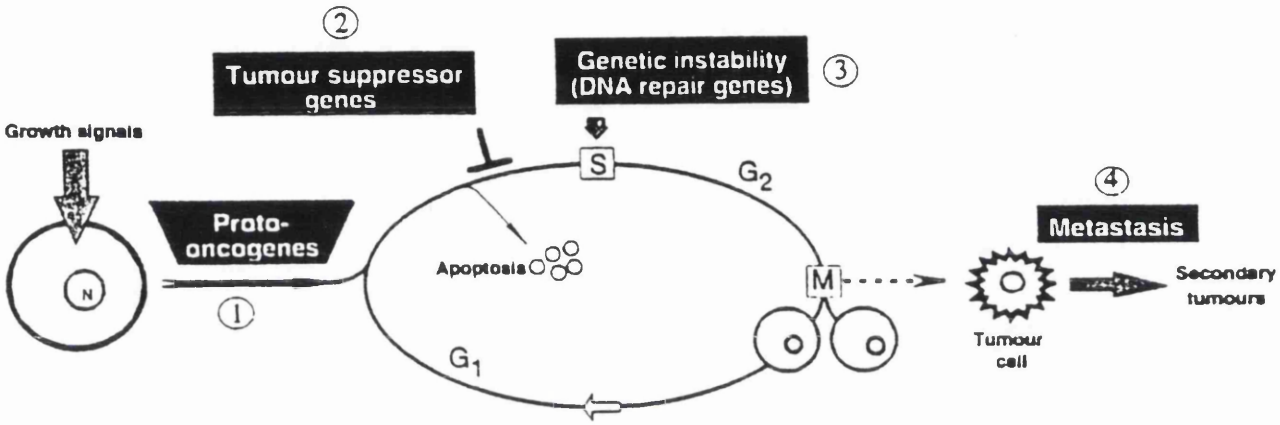


Fig 1.11 Genetic pathway in cancer development (adopted from Hesketh, The oncogene and tumour suppressor gene, 1995)

1.9.1 Cell cycle regulation and pathogenesis of cancer

Tumour progression is related to genomic changes represented by chromosomal aberrations, translocations and aneuploidy, which arise by cumulative genetic changes resulting from loss of cell cycle control. In other words, cancer can be described as a disease of the cell cycle.

There are two major control points within the cell cycle- one near the end of G_1, known as the restriction point (G_1-S) and the other at the initiation of mitosis (G_2-M). During the G_1 phase, cells responding to extracellular signals progress to either continuing toward another division cycle or withdrawal from the cycle into a resting state (G_0). The decision about division occurs as cells pass a restriction point late in G_1, after which they become refractory to extracellular growth regulatory signals and then carry out the autonomous program that commits them to the division. Although G_1 progression can normally be stimulated by mitogens or blocked by antiproliferative cytokines, cancer cells are not affected by these controls and are likely to stay in cycle. Consequently because cell cycle exit can facilitate maturation and terminal differentiation, these processes are also destroyed. The usual function of the G_2-M control point seems mainly to confirm that the DNA is completely replicated, undamaged and ready to be separated at mitosis (Carr, 1996; Sherr, 1966).

The progression of the cell cycle is checked for DNA damage at the G_1-S and G_2-M boundaries. Normal cells with DNA damage remain at either of these checkpoints during appropriate DNA repair. At the G_1-S checkpoint any damage sustained by cellular DNA is repaired with great fidelity. The second checkpoint occurs at the G_2-M boundary where cells can be arrested if they have damage not previously corrected after entering into the S-phase.

Many of the proteins that coordinate and control the cell cycles are identified. Various cyclin-dependent protein kinase complexes act as key regulators of cell cycle progression at the different stages of the cell cycle to integrate the independent events that constitute the cell cycles. In mammalian cells, a series of signalling pathways interact with the cyclin-dependent kinase and hold or stimulate entry into the cell cycle when it is appropriate. When errors develop,

either in the genetic material itself or through a conflict in signalling processes, eukaryotic cells are blocked up advancing through the cell cycle or lost completely by programmed cell death (Carr, 1996).

Activating mutations in oncogenes and inactivating mutations in tumour suppressor genes induce carcinogenesis. Activated oncogenes ultimately promote activation of CDK complexes and subsequently uncontrolled cell proliferation occurs. The checkpoint pathways become useless by inactivating mutations in tumour suppressor genes.

1.9.2 Oncogenes

Some tumour viruses such as retroviruses contain genes that are able to cause tumours. These genes are called viral oncogenes, v-oncogenes. In the human genome, there are normally genes with DNA sequences very closely related to the viral oncogenes.

Proto-oncogenes are normal cellular genes, which encode protein involved in normal cell division and differentiation (Bradley et al., 1995). The functions of protein products of proto-oncogenes in normal cells may include growth factors, growth factor receptors, signal transducers, and nuclear proto-oncogenes and transcription factors (Hodgson and Maher, 1993; Hesketh, 1995).

When a proto-oncogene becomes mutated, it may function as an oncogene. There are several mechanisms to induce mutation of proto-oncogenes: translocations, point mutations, deletions, and gene amplification. An oncogene is a gene whose action stimulates uncontrolled cell proliferation by losing control of normal cell growth and division.

1.9.3 Tumour suppressor genes

In normal cells there are gene products that have the ability to suppress the uncontrolled cell proliferation which is characteristic of malignant cells. The genes involved are called tumour suppressor genes, recessive oncogenes or growth suppressor genes.

While the normal products of tumour suppressor genes possess an inhibitory function in cell growth and division, this function is lost when tumour suppressor genes are inactivated and consequently this leads to unprogrammed cell proliferation.

Although inactivation of tumour suppressor genes has been linked to the development of a wide variety of human cancer, the two best understood tumour suppressor genes are the retinoblastoma (*RB1*) gene and *TP53*. Retinoblastoma provides the classical model for a recessive tumour suppressor gene in that both paternal and maternal copies of *RB1* must be inactivated for tumour to develop. For *TP53* and some other tumour suppressor genes, mutation at one allele may be sufficient to give rise to the altered cell phenotype (Hodgson and Maher, 1993).

1.9.4 Apoptosis

Apoptosis, or programmed cell death, is a natural physiological process which normally controls the lifespan of cells, occurring in embryonic development, differentiation and morphogenesis. It acts as a pathway of control of growth by a selective elimination of cells as another pathway of regulation of growth control. Apoptosis is also induced by exposure of cells to various agents and aberrant physiological conditions.

Cells undergoing apoptosis are characterized by distinctive morphological changes involving the condensation of nuclear chromatin and shrinkage of the cytoplasm, and the fragmentation of nuclear DNA. The apoptotic bodies are rapidly phagocytosed by neighbouring cells but this is not accompanied by an inflammatory response. The major biochemical feature of the apoptotic process is the fragmentation of nuclear DNA by Ca^{2+} -Mg^{2+} - dependent endonucleases which cleave the DNA at internucleosomal sites (Cohen and Duke, 1984; Caron-Leslie et al., 1991).

Several genes that are closely related with cell proliferation and neoplastic development are also associated with the apoptotic pathway. This indicates that the regulation of these processes may be interlinked and deregulation of

apoptosis may induce tumorigenesis and deregulation of cell cycle control would affect the cells into apoptotic pathway.

1.9.5 DNA Repair

DNA repair mechanisms are present to correct DNA damage due to environmental mutagens and accidental base misincorporation at the time of DNA replication. Inherited defects of either system result in an increased frequency of cancer (Connor and Ferguson-Smith, 1993).

The recognition and repair of DNA damage occurs before the cells enter the S-phase of the cell cycle and cells are held in the G1 phase until the repair process is completed.

Replication errors which are not corrected by the proofreading activity of DNA polymerase may be amended by a process called mismatch repair. This process includes the recognition of mismatched bases or single base insertion or deletions in the newly replicated strand followed by correction of the error. Four human DNA mismatch repair gene loci in humans have been identified, *MSH2*, *MLH1*, *PMS1*, *PMS2* (Hesketh, 1995) and these gene products act with each other to achieve mismatch repair in normal cells. Mutations in mismatch repair genes result in an overall increase of the mutation rate and predispose to cancer development.

1.9.6 Double minutes (DMs) and Homogeneously staining regions (HSRs)

These structures are amplifications of specific genetic material and are generally correlated with resistance to various drugs especially anti-metabolite drugs. They are also associated with many human cancers, frequently appearing in tumour cell lines, and are particularly associated with the progression of many tumours from benign to highly aggressive (Alitalo and Schwab, 1986).

Double minutes (DMs)

DMs are small spherical structures like minute chromosomes that each consist of approximately 1000-2000 kb of DNA. They do not participate in regular division at mitosis, segregating in a random fashion and producing an unequal distribution in the daughter cells (Clark and Wall, 1996).

Homogeneously staining regions (HSRs)

HSRs are large regions of non-banding amplified material within a chromosome. HSRs stain uniformly with G or Q banding with intermediate range of intensity. The size of the HSR is related to the length of the sequence amplified and the number of times it is multiplied. Multiple HSRs can occur in cells as a result of several amplification events or breakage and translocation of the original HSR.

1.9.7 Chromosomal Abnormalities in Malignancies

The probability that chromosome abnormalities were a cause of malignant transformation was suggested by Boveri in 1914 (Therman and Susman, 1993; Hodgson and Maher, 1993). Nowell and Hungerford identified the Philadelphia chromosome in chronic myeloid leukemia as the first consistent chromosomal abnormality in 1960. Then a large number of numerical and structural chromosome aberrations were discovered in wide range of cancers. Wide spectrum of chromosomal aberrations were found including numerical changes (from near haploid to polyploid, monosomy or trisomy), structural changes (partial monosomy or trisomy, unbalanced or balanced translocations, isochromosomes, isodicentrics, ring chromosomes), amplications (HSR, DMs), and marker chromosomes.

Although cytogenetic information from solid tumours was scarce because of technical difficulties, recent advances in culture techniques and in molecular-cytogenetic techniques has made possible remarkable progress in the analysis of solid tumours.

Chromosome aberrations in human cancer may be categorized into primary and secondary events. Primary chromosome aberrations occuring early in the development of the tumour are directly related to the genesis of cancer and are frequently found in particular tumours as a sole abnormality and show a highly nonrandom nature. Secondary chromosome aberrations tend to show more than one abnormality and are accompanied by primary changes. Secondary abnormalities may be additional phenomena during the progression of tumour or may have a role in the subsequent biological behaviour of the tumour, including invasion, metastasis and response to therapy (Hodgson and Maher, 1993).
Chromosome aberrations such as deletions of part of or an entire chromosome may induce inactivation of tumour suppressor genes. Translocations may activate an oncogene or inactivate a tumour suppressor gene. Gains of chromosomal material can lead to over-expression of oncogenes (Hodgson and Maher, 1993).

Leukemia

Leukemia accounts for about 2% of all human cancers and shows two peaks in the age incidence- in childhood and in the elderly (Hodgson and Maher, 1993). Despite the increased incidence of leukemia in congenital syndromes, most leukemia is likely to be an acquired condition. Most cases of leukemia reflect a stem cell disorder and the pathogenesis is often multi-step. Etiology is usually unknown, but may result from the interaction of endogenous factors such as inherent chromosome instability, abnormal DNA repair and altered immune function or exogenous factors such as radiation, chemicals and viruses.

i) Cytogenetic aspects of acute lymphoblastic leukemias

The identification of the chromosomal aberrations in acute lymphoblastic leukemia is particularly important to establish the exact diagnosis, to predict prognosis and to monitor the effects of therapy or bone marrow transplantation.
Clonal chromosomal abnormalities at diagnosis have been reported in between

44% and over 90% of patients analysed. The success of a cytogenetic analysis for detection of abnormal clone is dependent on optimal sample and culture conditions (Pui et al., 1990; Secker-Walker, 1994).

Importance of cytogenetics in ALL still continues as an independent prognostic indicator in spite of continuous adjustment to treatment regimens. An ever-increasing understanding of the genetic consequences of translocations and other chromosomal aberrations is extending the role of metaphase and interphase cytogenetics both in patient management and in increasing our understanding of the biology of the disease.

The convergence of cytogenetics and molecular techniques has now become a powerful method for understanding the events that predispose to, and result in, human ALL (Look, 1985).

ii) Established chromosomal aberrations of ALL

There are now at least 36 structural and 8 numerical abnormalities established in association with ALL (Secker-Walker, 1994).

Although the incidence of chromosomal aberrations may vary according to the patient's age, geographic location and the exact nature of the leukemia examined, the most common changes are t(9;22), t(4;11), and del(6q) followed by t(8;14), t(1;19) and del(9p) (Downing and Look, 1995).

a) Structural abnormalities

ALL with t(4;11)(q21;q23) is often observed in patients with ALL-L1or L2, is sometimes associated with congenital leukemia and has a poor prognosis similar to ALL with t(9;22). ALL with t(9;22) is observed in 6% of childhood and 17% of adult ALL. It is of pre B-cell or B-cell lineage and has an poor prognosis. B-cell ALL with t(8;14), t(2;8), or t(8;12) also has a very unfavourable prognosis and high incidence of CNS involvement at presentation possibly because of the very high cell proliferative rate of this disease (LeBeau, 1991; Kurzrock and Talpaz, 1995; Look, 1995). Other structural abnormalities including t(1;19),

t(8;22), del(6q), del(9p), t/del(12p) have not been demonstrated to have any prognotic significance (Secker-Walker, 1994).

b) Numerical changes - ploidy groups

Hypodiploidy;
Hypodiploidy, clonal loss of one or more chromosomes occur in 5%-8% of reported series in children and 9% of adults. There is little consistency in the clinical features of these cases. Prognosis for this group is variable.

Near-haploidy 23-36 chromosomes;
Near-haploidy is marked by the gain from haploidy (23 chromosomes) chiefly of chromosomes 10, 14, 18, 21 and the presence of both sex chromosomes, XX or XY. The reported incidence ranges from 0.7% to 2.4% and is more frequent in adolescent girls. This group has a particularly unfavourable prognosis with a median survival of 11 months.

Pseudodiploidy;
Pseudodiploidy describes a clone with a false dipolid complement of 46 chromosomes. The vast majority of pseudodiploid clones are accompanied by chromosomal translocations. Prognosis is usually unfavourable.

Low hyperdiploidy 47-50 chromosomes;
The most frequent gains are of chromosomes 21, X, 8 and 10 together with structural changes which often include abnormalities of 1q, 6q, 12p and 19p. The incidence in childhood leukaemia ranges from 11% to 15% and in adults between 8% and 11%. The prognosis is variable due to the heterogeneous nature of the group.

High hyperdiploidy 51-65 chromosomes;
Chromosomal gain in high hyperdiploidy is nonrandom with an excess gain of chromosomes X, 4, 6, 14, 17, 18 and 21. The incidence in childhood series (16%

to 27%) is at least 6 times greater than in adults (4%-5%). Usually patients with 51-60 chromosomes have had the most favorable prognosis (Pui et al., 1990; Van der Plas et al., 1992). There is some evidence that, within this subgroup, patients with trisomy 6 or with trisomies 4 and 10 have the best prognosis (Jackson et al., 1990; Harris et al., 1992). One characteristic which distinguishes patients with high hyperdiploidy is their response to the folate antagonist methotrexate (MTX) used in all treatment regimens. Improved survival has been demonstrated in patients whose lymphoblasts have the ability to accumulate high levels of methotrexate and methotrexate polyglutamate in vitro. This ability has been shown to be greatest in the lymphoblasts from children with hyperdiploidy (Whitehead et al., 1992). By combining metaphase cytogenetics with interphase cytogenetics using chromosome-specific centromeric probes, it becomes possible to demonstrate heterogeneity both in the mitotic index of the clone and in the elimination of the clone after 28 days of treatment. This technique will detect approximately 1/1000 clone cells.

Triploidy/tetraploidy;
Cases with >65 chromosomes represent a minority of cases in ALL with an incidence in childhood of approximately 1%.

Cytogenetic aspects of Acute myeloid Leukemia

AML is characterized by proliferation and accumulation of malignant myeloblasts and other immature myeloid cells. Infiltration of the marrow with these cells causes anemia, granulocytopenia and thrombocytopenia.
The classification of AML is based on the clinical presentation, cellular morphology, cytochemistry and immunology. The French-American-British (FAB) type, which is a morphological classification, serve as an important guide in prognosis and therapy of the individual leukemias and can act as a pointer to the likely chromosome abnormality which may be found.
Numerical or structural chromosomal aberrations in AML may occur alone or together. The frequency of chromosome abnormalities detected in AML at

diagnosis is variously reported as 60 to 90% depending on length of time in culture, banding techniques and referral patterns of reporting centres. With improved banding techniques, a chromosomal abnormality in at least 70% of cases could be detected (Walker et al., 1994).

The most frequently observed numerical abnormalities in all FAB types of AML are trisomy 8 and monosomy 7. Rarely observed are trisomies of chromosomes 4, 11, 13, 21 and 22. Numerical aberrations are seen in 15-20% of the cytogenetically abnormal AML cases (Downing and Look, 1995).

There are a number of common specific chromosome rearrangements which include translocations, inversions and deletions. t(8;21)(q22;q22) is the most common structural abnormalities in AML, seen in 10-15% of all M2 and some M4 cases, has a favorable prognosis with an uniformly high complete remission rate. t(15;17)(q22;q21) is detected in at least 70-90% of M3 cases and associated with good prognosis. inv(16)(p13;q22), which are associated with AML M4Eo. This subtype shows high complete remission rate with standard AML chemotherapy, which may be followed by subsequent CNS involvement, often with intracranial masses.

A poor prognosis is associated with several chromosomal aberrations in AML such as rearrangement of 3q21-q26, -5 or del(5q), -7 or del(7q), t(6;9), t(11q23), and del/t(12p).

Secondary AML

Secondary AML is usually defined as AML resulting from treatment with cytotoxic drugs and/or radiotherapy, or from exposure to environmental toxic agents. The most common abnormalities are –5/del(5q) and –7/del(7q) which are often present in the same clone. Other common cytogenetic aberrations are +8, -17, -18, +21, -21 and abnormalities of 3q, 6p, 11q, 12p, 17p and 21q. Complex and hypodiploid karyotypes are a common feature.

Well Differentiate Liposarcoma (WDLPS)

Liposarcoma is one of the most common types of soft tissue sarcoma in adults. WDLS is one of the histological subtypes of this malignant tumour including myxoid, round cell, and pleomorphic liposarcoma.

The characteristic cytogenetic findings of WDLPS are supernumerary rings, giant rod-like marker chromosomes and telomeric associations (Szymanska et al., 1996). These marker chromosomes may be derived material from chromosome 12q as well as material from chromosome 1, 4, 8 and 16 (Pedeutour et al., 1994). *SAS* and *MDM2*, well known to be amplified in liposarcomas, are located in chromosomal bands 12q13-14 (Nilbert et al., 1994; Pedeutour et al., 1994). In addition to amplification band on 12q14-21, chromosome 1q21-24 is often amplified (Szymanka et al., 1996).

Malignant Melanoma

Melanoma is one of the commonest human malignancies and one of the most malignant of all cancers. Epidemiologically, the incidence of melanoma is showing an increasing trend, especially among whites.

During early gestation, melanocytes migrate from the neural crest to the skin, mucous membranes, uveal tract and meninges. So melanoma can arise at any sites where tissue of neural crest origin is found and includes the epidermis, hair bulbs, leptomeninges and retina.

Most melanoma occurs in the skin. When it has invaded into the deeper dermis or fat, it metastasizes frequently to local regional lymph nodes or to other organs. In the early stages of melanoma, it is difficult to detect genetic alteration using molecular analysis because by current isolation methods it is difficult to obtain malignant cells without contamination with other cells. By several cytogenetic studies, malignant melanoma has shown to have structural abnormalities affecting chromosomes 1, 6, 7 and numerical abnormalities as +1, +5, +7, -9 and −10. In metastatic melanomas, additional nonrandom abnormalities have been shown on chromosomes 1, 2, 6, 7, 9, and 11 (Ozisik et al., 1994). By means of

cytogenetic studies of cutaneous malignant melanoma, 9p and 10q were seen to be involved in earliest stage of melanoma tumorigenesis although there is some controversy whether chromosome 10 is involved in the earliest stage or not. The incidence of loss of chromosome 10 (especially 10q) was very high. It suggests that putative tumour suppressor genes are probably located on chromosome 10q. It is of interest that loss of 10q has also been found in other neural crest derived tumours such as glioma (Karlborn et al., 1993; Indsto et al., 1998).

1.9 Aims of this project

The first aim of this project was to assess the usefulness of interphase FISH in the cytogenetic analysis of hematological malignancies, especially acute lymphoblastic leukemia, and then to evaluate whether CGH would be an approach to help identify chromosomal aberrations in hematological malignancies. The second aim was to assess the applicability of CGH to fresh frozen solid tumours. Finally, the aim was to optimise several procedures of whole genome amplification for analysing very small amounts of DNA using CGH, to evaluate the limiting amount of test DNA required for analyzable CGH images and to apply these optimized techniques on paraffin-embedded sections from samples of melanoma.

CHAPTER 2.

MATERIALS and METHODS

2. Materials and Methods

2.1 Safety

Laboratory coat and gloves were worn at all times during the experiments. Handling of human blood, bone marrow, and other biological materials was carried out in a appropriate safety cabinet. All glassware and where appropriate solutions were sterilized using an autoclave. If it was needed, some solutions except for primer, plastic equipment, microcentrifuge tubes, tips, and pipettors were exposed to UV irradiation. Toxic and carcinogenic agents were handled with care in a special fume hood according to safety guidelines.

2.2 Samples

There were two major sources of material for the investigations in this study. The first was the diagnostic laboratories of the Department of Medical Genetics. Blood and bone marrow samples remaining after diagnostic analysis were supplied by the cytogenetics laboratory as was the fresh frozen tissue from 5 cases of liposarcoma. The molecular genetics laboratory provided paraffin-embedded sections of ovarian tumour. The Cytogenetics laboratory also supplied the results of their diagnostic tests. In the later part of the study both fresh frozen samples and paraffin sections of melanoma were provided by the Dermatology Department of the Western Infirmary.

For the CGH study, control DNA was extracted from normal male and female peripheral blood.

2.3 Basic solutions for experiment

1. Tris-acetate (TAE, 50x)

   242 gram Tris base

   57.1 ml glacial acetic acid

   100 ml 0.5 M EDTA (pH 8.0), dissolve in 1000 ml of distilled water, store at room temperature

2. T.B.E. buffer (5x)

   54 gram Tris base

   27.5 gram Boric acid

   40 ml 0.5 M EDTA (pH 8.0), dissolve in 1000 ml of distilled water, store at room temperature

3. T.E. buffer (pH 8.0)

   10 mM Tris-HCl (pH 8.0)

   1 mM EDTA (pH 8.0)

4. Phosphate-buffered saline (PBS)

   10 Dulbecco PBS tablets in 1000 ml of distilled water, adjusted pH to 7.4 with HCl, sterilize by autoclaving and store at room temperature.

5. 1 M Tris HCl

   Dissolve 121.1 g of Tris base in 800 ml of distilled water, adjust the pH to the appropriate value by adding concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1000 ml with distilled water, and sterilize by autoclaving.

6. 5 M NaCl

   292.2 g of NaCl in 1000 ml of distilled water, sterilized by autoclaving

7. 0.5 M EDTA (pH 8.0)

Add 186.1 g of disodium ethylenediaminetetra-acetate.$2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH and sterilize by autoclaving.

8. 10% sodium dodecyl sulfate (SDS)

Dissolve 100 g of electrophoresis-grade SDS in 900 ml of distilled water. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 litre with distilled water. Dispense into aliquots.

9. 3 M sodium acetate

Dissolve 408.1 g of sodium acetate.$3H_2O$ in 800 ml of distilled water. Adjust the pH to 7.0 with dilute acetic acid. Adjust the volume to 1000 ml with distilled water. Dispense into aliquots and sterilize by autoclaving.

10. 10 M ammonium acetate (protein precipitating solution)

770 g of ammonium acetate in 1000 ml of distilled water, sterilize by filtration.

11. 20x SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of distilled water. Adjust the pH to 7.0 with a few drops of a 10 M solution of NaOH. Adjust the volume to 1000ml with distilled water. Sterilize by autoclaving. Store at room temperature.

12. 2x SSC

Dilute 20x SSC to 1/10 concentration, for example 50 ml of 20x SSC mix with 450 ml of distilled water. Store at room temperature.

13. 4x SSCT (4x SSC, 0.05% Tween-20)

    Add 250 µl of Tween-20 into 100 ml of 20x SSC, adjust the volume to 500 ml with distilled water. Store at room temperature.

14. 1 kb ladder

    100 µl of stock ladder solution are mixed with 200 µl of loading mix and 800 µl of distilled water.

15. Loading mix

    Mix 30% glycerol with 0.25% bromophenol blue in distilled water.

2.4 Chromosome preparation from cultured peripheral blood cells

2.4.1 Culture and Metaphase harvest of peripheral blood

Materials

Heparinized whole blood

Iscoves Medium

- 100 ml Iscoves medium (Imperial Labs Prod.)
- 1 ml Heparin (5,000 units/ml: LEO Laboratories)
- 1 ml Glutamine (Gibco BRL)
- 2 ml PHA (Gibco BRL)
- 1 ml Penicillin/Streptomycin (Gibco BRL)

10 µg/ml Colcemide (Gibco BRL)

75 mM KCl (0.56 g in 100 ml H_2O; store <2 weeks at room temperature)

Fixative: 3:1 absolute methanol/ glacial acetic acid

Procedure

1. Add 0.5 ml of heparinized whole blood to 4.5 ml of culture medium using an aseptic technique.
2. Mix the contents of each culture tube by gently inverting several times.
3. Incubate for 72 hours with tubes tilted at 45° in a humidified 37°C incubator.

Harvest culture

1. Initiate harvest by adding 0.1 ml of Colcemide. Mix by gently shaking the tube and incubate at 37°C for 45 minutes.
2. Centrifuge the tube at 1200 rpm for 7 minutes, room temperature. Discard the supernatant using pipette into a container of 10% Chloros.
3. Add 10 ml of 75 mM KCl (prewarmed at 37°C) and gently resuspend pellet.
4. Incubate the tubes at 37°C for 10 minutes.
5. Centrifuge the tubes at 1200 rpm for 7 minutes. Then discard the supernatant into a container of 10% Chloros.
6. Resuspend the pellet by adding 10 ml of fresh fixative.
7. Centrifuge the tubes at 1200 rpm for 7 minutes and remove the supernatant
8. Resuspend the pellet in 10 ml of fixative. Centrifuge the tubes at 1200 rpm for 7 minutes and pipette off the supernatant.
9. Resuspend the pellet by adding 5 ml of fresh fixative and place the tubes in the refrigerator (+ 4°C) for a minimum 30 minutes.
10. Centrifuge the tubes at 1200 rpm for 7 minutes and pipette off the supernatant.
11. Remove the supernatant and resuspend the pellet in 0.5 to 1.0 ml of fresh, cold fixative to produce a light milky suspension.
12. Prepare slides and analyse chromosome spreads.

2.4.2 Culture and Harvest by chromosome elongation methods

Materials

Culture medium

100 ml Iscoves medium (Imperial Labs Prod.)

1 ml Heparin (5,000 units/ml)

1 ml Glutamine (Gibco BRL)

2 ml PHA (Gibco BRL)

1 ml Penicillin/Streptomycin (Gibco BRL)

10μg/ml Colcemid solution

37.5 mM KCl (hypotonic solution)

Thymidine solution (30 mg/ml: Sigma)

10 ml distilled water

300 mg Thymidine

2-deoxycytidine stock solution (2.27 mg/ml: Sigma)

20 ml distilled water

45.4 mg 2-deoxycytidine

Store frozen as 1 ml aliquots

2- deoxycytidine working solution (0.227 mg/ml)

9 ml distilled water

1 ml 2-deoxycytidine stock solution

Procedure

1. Add 1.0 ml heparinised whole blood to 9 ml of culture medium using an aseptic technique
2. Incubate for 48 hours with tubes tilted at 45° in a humidified 37°C, 5% CO_2 incubator.

3. Add 0.1 ml thymidine solution to each 10 ml culture tube. (final concentration: 0.3 mg/ml)
4. Incubate the cultures for a further 18 hours in same conditions as above.
5. Add 0.1 ml of 2-deoxycytidine working solution to each 10ml culture. (final concentration: 10 μmol/ml)
6. Incubate the cultures for a further 3 hours and 55 minutes at 37°C.

Harvest culture

1. Add 0.1 ml colcemid to each 10 ml culture, mix by gently shaking the tube and incubate for an additional 20 minutes. (final concentration: 0.1 μg/ml)
2. Centrifuge the tubes at 1200 rpm for 7 minutes.
3. Discard the supernatant into 10% Chloros, leaving approximately 1 ml and resuspend the pellet by gently tapping the side of the tube.
4. Add 9 ml of 37.5 mM KCl solution (pre-warmed to 37°C) into above resuspended pellet. The first 0.5 ml should be added drop by drop whilst gently tapping the side of the tube.
5. Centrifuge the tubes at 1200 rpm for 7 minutes.
6. Discard the supernatant into 10% Chloros, leaving approximately 1 ml and resuspend the pellet by gently tapping the side of the tube.
7. Add 9 ml of cold, fresh fixative. The first 0.5 ml should be added drop by drop whilst gently tapping the side of the tube.
8. Centrifuge the tubes at 1200 rpm for 7 minutes and discard the supernatant.
9. Repeat steps 7 and 8.
10 Resuspend the pellet in 5 ml of cold, fresh fixative and place the tubes in the refrigerator (+ 4°C) for a minimum of 30 minutes.
11. Centrifuge the tubes at 1200 rpm for 7 minutes and discard the supernatant.
12. Add 0.5 to 1.0 ml of cold, fresh fixative solution to resuspend the pellet.

2.4.3 Slide Preparation

1. Clean microscope slides by soaking in Decon overnight and rinse with running tap water for at least 2 hours. Stored the clean slides in 70% ethanol.
2. Remove the slides from ethanol and rinse with running water until the ethanol has gone and a thin, uniform film of water cover the slides. Keep the slides inside water at + 4°C at least for 30 min.
3. Centrifuge the tubes containing the cell suspension at 1200 rpm for 8 minutes and discard the supernatant, resuspend the pellets in 0.5-1 ml of fresh cold fixative solution. Place 3-4 drops of the cell suspension onto cold, grease free, wet slides.
4. For G-banding, place the slides on a hotplate to dry and evaluate under a light microscope (phase contrast). For FISH or CGH, allow the slides to air dry.

2.5 Chromosome banding technique

2.5.1 Giemsa banding

Materials

Sorensons Buffer

1000 ml of distilled water

9.48 g Disodium dihydrogen phosphate

9.08 g Potassium dihydrogen phosphate

Trypsin solution

1000 ml of Sorenson's buffer

1.2 g Difco Trypsin (1:250)

Staining solution

2000 ml of methanol

3 g of Leishmans powder

Filter this solution before use. For staining purposes dilute this solution with buffer (pH 6.8) in the ratio 1 part stain to 3 parts buffer.

Procedure

1. Age the slides by placing them in a dry oven at 80°C for two hours.
2. Immerse the slide in trypsin solution solution for 10 to 15 seconds.
3. Rinse with saline and stain with Leishmans solution for two minutes.
4. Place the slide on the hotplate to dry.

2.6 Fluorescence In Situ Hybridisation

In this study, test materials for FISH were peripheral blood or bone marrow, paraffin-embedded tissue. In the early part of this study, FISH was performed with chromosome specific centromeric probes and whole chromosome libraries to become familial with FISH technique, then mono- or dual colour FISH using alpha-satellite, chromosome-specific probes was carried out for the study of hematologic malignancies. Finally FISH was performed on paraffin-embedded tissue using unique sequence probes.

2.6.1 Labelling of Probes by nick translation

In the first part of this study probes were labelled indirectly with biotin or digoxigenin where probes were commercially unavailable.

In this method, digoxigennin or biotin were used as reporters instead of directly fluorochrome coupled nucleotides. Biotinylation was carried out by nick translation using biotin-11-dUTP.

Material

A) For nick translation

Biotin-dUTP or Digoxigenin-dUTP (Sigma)

DNA to be labelled

Nick translation system (Gibco BRL)

B) For ethanol precipitation

3M sodium acetate

Glycogen (Boehringer Mannheim)

80% cold ethanol (filtered)

Procedure:

1. Prepare the solution to a final volume of 50 µl solution containing 1 µg of the DNA to be labelled, 5µl of solution A4 (dATP, dCTP, dGTP), 3 µl of biotin-11-dUTP, 5 µl of DNA polymerase 1/DNase 1 enzyme mixture, and distilled water.
2. Mix the content by inverting the tube and incubate at 15°C for 90 minutes.
3. Stop reaction by adding 5 µl of stop buffer (solution D).

Ethanol precipitation to remove unincorporated nucleotides

4. Add 5 µl of 3 M sodium acetate, 1 µl of glycogen, and 122µl of ice-cold 85% ethanol to the probe.
5. Mixed the content by inverting the tube, placed at -70°C for 30 minutes or –20°C for overnight and then centrifuge at 14000 rpm for 30 minutes.
6. Discard the supernatant.
7. Allow the pellet to air dry, and then dissolve in 20 µl of 1x TE buffer.

For labelling with digoxigenenin, all procedures are the same as for biotinylation except for substitution of 3µl of biotin-11-dUTP for 2µl of digoxigenin-dUTP.

2.6.2 FISH to metaphase chromosomes, interphase nuclei

Materials

A) For preparation of target metaphase and probe

Slides containing metaphase chromosomes or interphase nuclei of interest

Non-isotopically labelled DNA probes (chromosome specific libraries, centromeric probe)

Hybridisation buffer (50%formamide/2x SSC)(Hybrisol V11; Oncor)

B) For denaturation of target DNA

Denaturation solution: 70% (v/v) formamide/2xSSC (pH 7.0)

50%, 70% (ice-cold), 70%, 90%, 100% ethanol

Water bath (70°C)

C) For Hybridisation

Water bath (42°C)

22-mm^2 coverslips

Rubber cement

D) For Post-hybridisation washing and detection

50% (v/v) formamide/2x SSC

2 x SSC

4 x SSCT

Blocking agent (850µl of 4 x SSCT + 150µl of 15% human AB serum; Sigma)

Biotin detection: Antiavidin -FITC, Avidin –FITC (Vector Laboratories)

Digoxigenin detection: Anti-digoxigenin FITC, Anti-sheep FITC (Vector Laboratories)

70%, 90%, 100% ethanol

DAPI or propidium iodide staining solution (Sigma Chemical Co)

Appropriate antifade mounting solution (Citiflour)

Water baths (42°C, 37°C)

22-mm^2 coverslips

Nail polish

E) Visualization

Epifluorescent microscope with appropriate filter

CCD camera and computer with Smartcapture software

Procedure

A) Preparation of target metaphases

1. Examine the slide, select the region with best metaphases and mark the slide using diamond pen.
2. Dehydrate the aged slide by soaking in the series of ethanol(50%-70%-90%-100%) for 2 minutes

B) Denaturation of DNA in target metaphases and probe

3. Soak slide containing metaphase chromosomes for 3 min in 70% formamide in a Coplin jar in a 70°C water bath.
4. Rinse slide for 2 min in ice-cold 70% ethanol in Coplin jar to stop denaturation. Then continue dehydration by soaking slide for 2min in room temperature in 70%, 90%, and 100% ethanol. Allow the slide to air dry.
5. Check denatured chromosome preparation with a phase-contrast microscope.
6. If probe is a centromeric probe, 1-1.2 µl of probe is mixed with 10 µl of hybridisation buffer. If probe is whole chromosome library, 15 µl of probe is used.
7. Heat for 10 min at 70°C to denature the probe. Then, if probe is a centromeric probe, an suppression step is not needed. If probe is a whole chromosome library, incubate the probe for 1 hour at 37°C.

C) Hybridisation

8. Place denatured probe mix on marked area of target metaphase slide. Cover

with a 22 mm^2 coverslip, remove any air bubbles with gentle pressure, seal with rubber cement, and incubate overnight (about 16 hrs) in a water bath at 42°C to hybridise.

D) Post-hybridisation washing and detection

9. Remove the coverslip by soaking in 2 x SSC carefully, having first removed the cement. Wash the hybridized slide in two changes of 42°C pre-warmed 50% formamide/2x SSC wash solution 5 minutes each, in 42°C 2 x SSC 5 minutes twice, then in room temperature 4 x SSCT for 5 minutes once.
10. Take off slide, place it flat and blot excess buffer. Do not allow slide to dry at any point in the procedure.
11. Add 100 µl of blocking solution to the slide and cover with parafilm. Incubate for 15 minutes on a tray in a water bath at 37°C.
12. Add 100 µl of detecting reagent to hybridized chromosome preparation on slide and cover with parafilm. Incubate for 30 min on a tray in a water bath at 37°C.

Preparation of detecting reagent:

In cases with biotin labelled probe:
498 µl of blocking agent + 2 µl of fluorescence Avidin
496 µl of blocking agent + 4 µl of fluorescence Anti-Avidin
Mix above solutions in two separate tubes, leave at room temperature for 10 min, spin at 14000 rpm for 10 min, mix supernatant together in one tube, and incubate in a 37°C water bath at least 15 min.

In cases with digoxigenin labelled probe:
Mix 500 µl of blocking agent + 0.75 µl of Anti-digoxigenin FITC + 0.75 µl of Anti-sheep FITC in one tube, leave at room temperature for 10 min, spin at 14000 rpm for 10 min, transfer supernatant to another tube and incubate in a 37°C water bath at least 15 min.

In cases with biotin/digoxigenin labelled probe (dual colour):

Mix 995 μl of blocking agent with 2.5 μl of Avidin FITC and 2 μl of Anti-digoxigenin rhodamine, continue with remaining procedure as digoxigenin. In this case, there is no need for incubation.

13. Rinse slide sequentially in Coplin jars containing 4 x SSCT at room temperature for 3 min three times. Then continue dehydration by soaking slide 2min in room temperature 70%, 90%, and 100% ethanol and allow slide to air dry.
14. Add 10 μl of DAPI (0.1μg/ml) with antifade solution to stained slide and add a coverslip. Seal with nail polish.

D) Visualization

15. Examine the slide using a fluorescence microscope with an appropriate filter set with or without a CCD camera and computer equipped with SmartCapture software system.

2.6.3 FISH to paraffin-embedded tissue

Materials

Paraffin-embedded tissue of interest

Histoclear

50%, 70%, 90%, 100% ethanol

Protein digestion enzyme solution (from Tissue Kit of Oncor)

Tissue pretreatment solution (from Tissue Kit of Oncor)

1 x PBD

2 x SSC

10 to 12 ng biotin- or digoxigenin- labelled centromeric DNA probe

10 μl of unique sequence probe (p53 DNA probe, Oncor) or 1 μl of unique

sequence probe (LSI p53 Spectrum Orange, Vysis)
Hybridization solution: 70% (v/v) formamide in 2 x SSC

Procedure

A) Sample preparation

Tissue should be cut into 4 μm thickness sections and applied to silanized slides and after attaching sections to slide, allowed to air dry. The slides were then baked at 65°C for 6 hours.

In this study, these slides were prepared at the Dermatology department.

B) Tissue sample preparation

1. Place up to four slides in a Coplin jar containing 50 ml of fresh histoclear. Allow the slides to soak at room temperature for 15 minutes twice.
2. Transfer the slides to a Coplin jar containing 100% ethanol. Soak the slides for 10 minutes at room temperature. Pour off the ethanol and add fresh 100 % ethanol to the jar containing the slides and soak for another 10 minutes.
3. Remove the slides from the ethanol and allow to air dry.
4. Place up to four slides into pre-warm (45°C) pretreatment solution and incubate at 45°C for 10 minutes.
5. Rinse the slides by placing them in a Coplin jar filled with fresh 2 x SSC and agitating for 5 to 10 seconds
6. Place up to four slides into the pre-warm (45°C) protein digesting solution
7. Incubate the slides for 16 minutes at 45°C.
8. Rinse the slides by placing them in a Coplin jar filled with clean 2 x SSC and agitating for 5 to 10 seconds.
9. Dehydrate slides in 70%, 90%, 100% ethanol series at room temperature for 1 minute each. Allow slides to air dry.
10. Check the degree of pretreatment and protein digestion by applying with Propidium iodide counterstain.

C) Probe preparation

Co-hybridisation of satellite probe with unique sequence probe (Oncor; indirect labelled):

1. Combine 1µl of satellite probe and 9µl of hybridization buffer with 10µl of unique sequence probe and mix well.
2. Pre-warm probe mix for 5 minutes at 37°C.

Co-hybridization of satellite probe with unique sequence probe (Vysis; direct labelled):

1. Mix 1µl of satellite probe, 1µl of unique sequence probe, 1µl of distilled water, and 7µl of hybridization buffer.
2. Centrifuge shortly and vortex and centrifuge again.

D) Hybridisation

1. Place the probe mix on each slide, cover with a coverslip and seal with rubber cement.
2. Denature the probe and target DNA simultaneously by placing the slide in 74°C oven or slide warmer for 5 minutes.
3. Incubate slides overnight in a 37°C water bath.

E) Post- hybridisation wash and detection

Indirect labelled probe:

1. Pre-warm 50 ml of 2 x SSC in a glass Coplin jar to 72°C-74°C in a water bath.
2. Remove the coverslip carefully.
3. Immerse slides in pre-warmed 2 x SSC for 5 minutes.
4. Transfer slides to a Coplin jar containing 50 ml of 1x PBD for 2 minutes.
5. Remove slides from 1x PBD and blot excess fluid from the edge.
6. Apply 60 µl of blocking agent (same as FISH on metaphase) to each slide and place parafilm over the solution. Incubate slides for 10 minutes in a 37°C

water bath.

7. Apply 60 µl of the detection reagent to each slide and place a parafilm over the solution. Incubate slides for 15 minutes in a 37°C water bath. For detection reagent of dual colour, use same method as FISH on metaphase.
8. Carefully remove the coverslip and wash slides 3 times for 2 minutes each in 50 ml of 1x PBD at room temperature.
9. Remove slides from 1x PBD and blot excess fluid from the edge.
10. Apply 10 µl of the appropriate counterstain. Cover with a glass coverslip and seal with nail polish.
11. Evaluate slides with an epi-fluorescence microscope.

Direct labelled probe:

1. Pre-warm 50 ml of 0.4 x SSC with 75 µl of tween 20 in a glass Coplin jar in a 73°C water bath.
2. Remove the coverslip carefully.
3. Immerse slides in pre-warmed 0.4 x SSCT for 2 minutes.
4. Wash slides in 2 x SSC for 2 min.
5. Allow slides to air dry.
6. Apply 10 µl of the appropriate counterstain. Cover with a glass coverslip and seal with nail polish.
7. Evaluate slides with an epi-fluorescence microscope.

2.6.4 Microscopy and Image analysis

Light Microscope

Phase contrast microscope is used for identifying the best part of slide for FISH.

Fluorescence Microscopy

Fluorescence microscopy takes advantage of the fact that many dyes absorb light energy and subsequently emit part of this energy at a different wavelength.

Examination of signals is performed either by using 100x Plan-achromatic objective of epifluoresence microscope (Zeiss Axioplan) or by using epifluorescence microscope equipped with CCD camera and computer with SmartCapture software system.

Light sources

The light source for epi-fluorescence microscopy is a mercury lamp (50W or 100W).
Mercury bulb is changed every 200 hr because there is a risk of explosion that can damage the microscope.

Filters

Choice of filters for fluorescence microscopy is determined by the fluorochrome and counterstain used in sample preparation. Dual- and triple band-pass filters are used to permit simultaneous visualization of dual or triple fluorochrome combinations.

2.7 Comparative Genomic Hybridisation

2.7.1 Preparation of normal target metaphase slides

High quality of human metaphase preparations is critical step for successful result of CGH.
Metaphase spreads were prepared as previously mentioned in 2.4.3. Slides were kept at room temperature to dry and used after 1-2 days.
Although it was best to use metaphases from a male donor, sometimes the donor was matched to patient sex for further careful analysis of sex chromosomes.
Before using for CGH, over 100 metaphase slides were made at once to

minimize inter-experimental fluctuation and several slides from each batch were checked for high quality of metaphases. Differently labelled normal versus normal hybridisation was applied to a test slide from each batch of metaphase spread preparations. If quality of slides was acceptable, the remaining slides in the same batch were used for further CGH experiments. If not, the entire batch was discarded. After starting experiments with DNA from paraffin-embedded tissue, slides made commercially (Vysis Ltd) were used to increase objectivity.

2.7.2 Preparation of Genomic DNA for CGH

Isolation of genomic DNA from peripheral blood and bone marrow

Materials

RBC lysis solution
155 mM Ammonium Chloride
10 mM Potassium Hydrogen Carbonate
1 mM EDTA

Cell Lysis:
25 mM EDTA
2% SDS

Proteinase K (20mg/ml) (Gibco BRL)
10 mM Ammonium acetate
Isopropanol
Ethanol (70% v/v)
TE buffer

Procedure

1. Collect the blood or bone marrow into a vacutainer containing EDTA
2. Add 3 volumes of RBC lysis solution to one volume of blood or bone marrow sample.
3. Invert the sample several times to mix and keep at room temperature for 20 minutes before being centrifuged at 2000 g for 7 minutes (large sample) or at 14,000 g for 20 seconds (small sample).
4. Discard the supernatant until approximately10-20 µl liquid with white cell pellet remains.
5. Resuspend cell pellet with remaining liquid by vortexing, add 1 volume of cell lysis solution, and vortex again.
6. Add 1/3 volume of protein precipitation solution, vortex, and centrifuge at 3000 g for 20 minutes (large sample) or at 14,000 g for 3 minutes in microcentrifuge (small sample).
7. Transfer supernatant to clean tube containing 1 volume isopropanol and mix thoroughly to form DNA precipitate.
8. Spool out DNA using sealed Pasteur pipette, and wash in 70% ethanol. If the sample is small, spin down the DNA.
9. Dry the DNA pellet, and dissolve in various amount of TE buffer depending on size of pellet.

Isolation of genomic DNA from fixed cells

Procedure

1. Add 5ml of 2 x SSC to the fixed cell pellet to wash off the methanol/acetic acid and spin for 10 minutes at 1000 rpm.
2. Discard the supernatant and repeat step 1 again.
3. Discard the supernatant and add 5 ml of cell lysis buffer to remaining pellet.

Remaining steps (from No 6) are the same as above.

Isolation of genomic DNA from fresh frozen tissue

Material

Cell lysis solution
Proteinase K solution (20mg/ml, GibcoBRL)
RNase A solution (Sigma)
Protein precipitation solution (10 M Ammonium acetate)
Isopropanol
Ethanol (70% v/v)

Procedure

1. Chill a 1.5 ml tube containing 600 µl cell lysis solution on ice. The solution will turn cloudy.
2. Add 10-20 mg of fresh or frozen tissue to the cooled cell lysis solution, and quickly homogenize using pestle. Keep homogenized samples on ice.
3. Transfer lysate to 1.5 ml eppendorf tube and incubate lysate at 65°C for 15-60 minutes. Then add 3 µl Proteinase K solution (20 mg/ml) to the lysate and incubate at 55°C for 3 hours or until tissue has dissolved.
4. Add 3 µl RNase A solution to the cell lysate.
5. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15-60 minutes.
6. Cool sample to room temperature.
7. Add 200 µl 10 M ammonium acetate to the RNase A-treated cell lysate.
8. Vortex vigorously at high speed for 20 seconds to mix the 10mM ammonium acetate evenly with cell lysate.
9. Centrifuge at 14000 g for 3 minutes.

DNA precipitation

1. Transfer the supernatant containing the DNA into a clean eppendorf tube (1.5

ml) containing 600 µl 100% isopropanol.

2. Mix the sample by inverting gently 50 times until the white threads of DNA form a visible clump.
3. Centrifuge at 14000 g for 1 minute: the DNA will be visible as a small white pellet.
4. Discard the supernatant carefully, add 600 µl 70% ethanol, and invert the tube several times to wash the DNA pellet.
5. Centrifuge at 14000 g for 1 minute. Discard the ethanol taking care not to loose the DNA pellet.
6. Drain the tube on clean absorbent paper and allow sample to air dry.
7. Resuspend in TE buffer.
8. Keep DNA at room temperature overnight or heat at 65°C for 1 hour to dissolve DNA.

Isolation of genomic DNA from paraffin-embedded tissue

A) Method 1:

Materials

Histoclear
100% ethanol
Proteinase K (20mg/ml, Gibco BRL)
10 M Ammonium acetate
70% Phenol: chloroform (1:1) saturated in H_2O
TE buffer

Procedure

De-paraffinizing step:

1. Centrifuge tissue to the bottom of the 1.5 ml tube for 5 seconds.

2. Add 1ml of histoclear and gently vortex to loosen the paraffin from the bottom of the tube. Leave at room temperature for 10 minutes.
3. Centrifuge for 2 minutes at full speed in a microfuge.
4. Remove supernatant (histoclear) carefully.
5. Repeat steps 2-4.
6. Add 0.5 ml of 100% ethanol to the tube and mix well, centrifuge for 2 minutes and carefully remove ethanol.
7. Repeat step 6.
8. Air dry.

Proteinase K digestion:

1. Add 500 µl of lysis buffer (500 µl nuclei lysis buffer, 0.5% SDS) to the tube containing de-waxed tissue pellet and mix well.
2. Add 0.4 mg/ml Proteinase K and incubate at 55°C for 10 hr with frequent tapping.

Phenol/chloroform extraction:

1. Add I volume of 1:1 phenol/chloroform to the proteinase K digested DNA sample, and mix to form an emulsion.
2. Incubate at room temperature for 10 minutes.
3. Microcentrifuge for 2 min at maximum speed at room temperature.
4. Transfer supernatant (DNA in the top layer) to clean microcentrifuge tube.

Ethanol precipitation:

1. Add 1/3 volume of 10 M Ammonium acetate to the DNA sample to be precipitated.
2. Add 2 to 2.5 volume (calculated after addition of 10 M Ammonium acetate) of ice cold 100% ethanol. Mix well. Incubate 15 to 30 minutes in dry ice.
3. Microcentrifuge 10 minutes at maximum speed.
4. Discard the supernatant carefully, add 1 ml of ice-cold 70% ethanol. Mix and microcentrifuge for 5 minutes at maximum speed.

5. Allow the pellet to air dry.
6. Dissolve in TE buffer, pH 8.0.

B) Method 2

Deparaffinizing step:

Same as Method 1

Proteinase K digestion:

1. Suspend the samples in 200 μl DNA extraction buffer (0.3 mg/ml proteinase K, 100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8, 25 mmol/L EDTA pH 8, and 0.5% sodium dodecyl sulfate). Incubate at 55°C overnight.
2. Add additional proteinase K (5 μl from 20 mg/ml stock solution) 24 hours and 48 hours later for a total incubation time up to 96 hours.
3. Inactivate enzyme by heating at 94°C for 10 minutes or 85°C for 15 minutes.

Phenol/chloroform extraction:

Same as method 1

Ethanol precipitation:

Same as method 1

C) Method 3

Deparaffinization and Proteinase K digestion

1. Suspend the samples in 20 μl of 1x PCR buffer 11(10mM Tris-HCl, pH 8.3, 50mM KCL; Perkin-Elmer, Norwalk, CT, USA).
2. Heat at 95°C for 10 min under paraffin oil with periodic mixing to aid deparaffinization.
3. Add proteinase K (2mg/ml) to the samples and incubate at 65°C for 90 min, then inactivated at 85°C for 15 min.

4. Directly use crude DNA for whole genome amplification without phenol/chloroform extraction.

2.7.3 Preparation of labelled probes for CGH

Direct labelling method

Materials

1 μg of test or control DNA in 38 μl double distilled water
5 μl 10 x A4 mixture (0.2 mM dATP, dCTP, dGTP in 500 mM Tris-HCl(pH 7.8), 50 mM $MgCl_2$ 100 mM β-mercaptoethanol, 100μg/ml BSA)
1 μl (>1 nmol) FITC-12-dUTP (Dupont, Boston, MA) or Fluorescein-11-dUTP (Amersham) for test DNA
1 μl (>1 nmol) Texas Red-5-dUTP (Dupont, Boston, MA) or rhodamine-4-dUTP (Amersham) for control DNA
5 μl enzyme mixture containing DNA polymerase 1 (>2 U) and DNase 1 (>200 pg) (Gibco BRL, Gaithersburg, MD)
1 μl (>10U) DNA polymerase 1 (Promega, Madison, WI)

Procedure

1. Mix all above solutions in a microcentrifuge tube.
2. Incubate 45-90 minutes at 15°C depending on quality of DNA.
3. Stop reaction by 10 minutes incubation at 65°C.
4. Run a double stranded 1% agarose gel to check the probe fragment distribution. Adjust incubation time and amount of enzyme mixture for obtaining ideal size of fragment (600-2000 bp)

Indirect labelling method

Materials

1 μg of test or control DNA

10 x A4 mixture; 0.2 mM each of dATP, dCTP, dGTP, in 500 mM Tris-HCL (pH 7.8), 50mM $MgCl_2$, 100mM 2-mercaptoethanol (Gibco BRL)

0.4 mM biotin-11-dUTP or 0.35 mM digoxigenin-11-dUTP

DNA polymerase 1/DNase 1 mix: 0.5 U/ul DNA Polymerase1, 0.4 mU/μl DNase 1 in 50 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 0.1 mM PMSF, and 50% (v/v) glycerol, 100 μg/ml nuclease-free BSA

Stop buffer: 0.5 M EDTA (pH 8.0)

Procedure

1. Mix with 5 μl of A4 solution, 1μg of DNA, 3 μl of biotin-11-dUTP or 2 μl of digoxigenin-11-dUTP, add water up to 45 μl of total volume.
2. Add 5 μl of DNA polymerase 1/DNase 1 mixture, mix gently, and centrifuge briefly.
3. Incubate at 15°C for 90 minutes
4. Add 5 μl of Stop buffer or heat at 68°C for 10 minutes.

2.7.4 Preparation of probe mixture for hybridization

Materials

labelled test DNA

labelled control DNA

Cot-1 DNA (1μg/ μl, Gibco-BRL)

3 M sodium acetate

100% cold ethanol

Hybridisation buffer (50% formamide, 10% dextran sulfate, pH 7, Phamacia)

Procedure

1. Mix 400-800 ng of test DNA, control DNA, and 20-40 μg of human Cot-1 DNA in a 1.5 ml microcentrifuge tube. In test DNA from fresh samples, equal amounts of test and reference DNA were used. In test DNA from paraffin-embedded tissue, test DNA was used 2 or 2.5 fold of reference DNA and amount of human Cot-1 DNA was also increased.
2. Add 1/10 volume of 3 M sodium acetate, mix, then add 2.5 volume of 100% ethanol and mix again for precipitation of DNA.
3. Microcentrifuge 30 minutes at maximum speed.
4. Discard the supernatant and vacuum dry for 15 minutes.
5. Add 10 μl hybridisation buffer (50% formamide, 10% dextran sulfate, pH 7) and resuspend pellet gently.
6. Denature the probe mix for 5 minutes at 70°C immediately before use.

2.7.5 Preparation of target metaphase slides

1. Mark the area of the slide containing metaphases with a diamond pen.
2. Denature metaphase slides for 3 minutes at 72°C-73°C in a denaturation solution (70% formamide, 2x SSC, pH 7).
3. Plunge slides into 70% ice-cold ethanol.
4. Dehydrate in a series of ethanol (70%, 90%, 100%), 2 minutes each.
5. Allow slides to air dry.

Optimisation of denaturing time for CGH was checked after denaturation step at the beginning of each new batch of slides.

2.7.6 Hybridisation, Post-hybridisation washing, and Detection

Direct labelling method:

1. Apply 10 μl of hybridisation mixture to the slide, cover with a 22mm^2 coverslip and seal with rubber cement.
2. Incubate for 2-3 days in a 37°-40°C water bath.
3. Remove coverslip and wash slides twice in a washing solution (50% formamide, 2x SSC, pH7) at 45°C for 15 minutes each.
4. Wash in 2 x SSC at 45°C for 5 minutes once.
5. Wash in 1 x SSC at 45°C for 5 minutes once.
6. Wash once in 4 x SSC at 45°C and again at room temperature, 5 minutes each.
7. Dehydrate in a series of ethanol (70%, 90%, 100%), 2 minutes each.
8. Allow slides to air dry and mount in an antifade solution containing 0.1-0.2 μM DAPI.

Indirect method:

Same as washing and detection method with dual-colour FISH

2.7.7 CGH with inverse labelling

Usually, test DNA is labelled with FITC-12-dUTP, control DNA with TexasRed-5-dUTP. In case of reverse CGH, test DNA is labelled with TexasRed-5-dUTP, control DNA with FITC-12-dUTP.

2.7.8 Microscopy, Imaging, and Image analysis for CGH

Direct visual inspection

Metaphases spreads hybridised by CGH are examined using an epifluorescence microscope with 100x oil-immersion lens and appropriate filter sets for the

fluorochromes used. Differences in relative intensities of the two fluorochromes can be visualized by using a dual-band-pass filter that transmits red and green fluorescence simultaneously, along with a filter for the DAPI counterstain. Although certain sequence copy number changes such as gene amplification (> 10 to 20 fold) and changes that include a large region may be visually detected, small regions of gain or deletion (<10 to 20 Mb) may not be visible to the human eye.

Microscope

Epiflurescence microscope equipped with high-numerical-aperture, plan-corrected, and chromatically corrected objectives are used for acquisition of high-quality images with minimal aberrations.

Optical filter

Appropriate filters in which are matched to the fluorochromes are used.

Camera

For image acquisition, epifluoresence microscope (Axioplan: Zeiss, Germany) equipped with a cooled CCD camera (Photometrics) and computer (Mackintosh) was used. This camera was controlled by an image analysis system installed in computer.

Digital image analysis

A) Image capture

Capturing of image was performed using an epifluorescence microscope (Axioplan: Zeiss, Germany) equipped with a cooled CCD camera (Photometrics) controlled by SmartCapture™ V2.1 software (Digital Scientific, Cambridge, UK).

1. After identification of ideal metaphase using DAPI filter under the epifluorescence microscope, activate **Alias of IP Lab** in the Apple Menu items.
2. Select **Capture** from the Extension Menu.
3. Click **More Options** and set up optional menu (normalization, exposure time, gain, binning), camera, and filter.
4. Click **Done** to close the window for remembering this setting.
5. Select **Expose** and then the three excitation frequencies yellow (rhodamine), blue-green (FITC) and blue (DAPI) appeared sequentially. Composite image from the three colour components and colour normalisation window came up on the screen after a brief pause.
6. Same Normalisation setting was kept on during analysis of the same patient sample.
7. For saving image, choose **Save As** with PICT format in a allocated folder.
8. Five to ten images were captured and saved in the same way.

B) Image analysis

For proper CGH analysis, 7 steps of analysing procedure were performed in addition to assessment of image quality.

After image capture, image analysis was performed two ways-SmartCapture system and Quips CGH analysis system. By using SmartCapture system, all procedures were performed manually, while using Quips CGH analysis system the procedures were carried out automatically.

C) SmartCapture System

i) Correcting the background

For correct quantitation, CGH images are first background corrected by applying a top hat filter.

1. Select **Background Remove** from the Extension Menu and set filter size to **27** on red, green and blue filter with **better quality**.
2. Select **New Window** and click **OK**.

ii) Segmentation of image

Software used in this study produced a segmentation mask where each pixel was identified as a foreground or background pixel and contiguous clusters of foreground pixels make up chromosomes.

1. Choose **Script for segmentation** in script folder and then select **Segmentation** from Analyse Menu. Check the **binarise box**, **green inside** and choose **Show ROI histogram**.
2. By moving line, choose a value of size and extent of the chromosomes.
3. Click on **Continue** after recovering the script.

iii) Identification of chromosomes

Identification of specific chromosomes is possible visually based on the R-banding pattern in the DAPI image. Identification can be facilitated using gray-value morphology filtering to enhance the banding pattern and by displaying using an inverted lookup table.

iv) Normalisation of the fluorescence intensity

It is necessary to normalise the intensities of the hybridisation signals to calculate ratios and to combine information from different metaphase spreads. Normalisation is accomplished by equalising median intensities of the test and reference DNA images.

Normalisation is achieved after running **Script for colour (fluorescence) equalisation** in script folder.

v) After normalisation, detection of relative copy number changes can be detected by Global method and Axial analysis. In the Global method, individual metaphases are examined at the level of individual picture elements (pixels) and calculating fluorescence ratio and coding this to various colours according to the magnitude (FR >1; green represents gain, FR=1; white balanced state, FR<1; red represents loss). Global method is useful to detect hypotriploidy/tetraploidy. Axial method is replaced by automatic Quips CGH analysis program.

D) Quips CGH analysis system

In this system, usually Quips CGH/Karyotyper and Quips Interpreter were used. Quips CGH/Karyotyper is for editing and analysing a metaphase image to generate a karyotype and CGH ratio profiles. Quips Interpreter is for editing and analysing ratio profile data from several metaphases in a CGH experiment. Sequence of analysis and features of this system are presented in Fig 2.7.1 and Table 2.7.1.

i) Image analysis by using Quips CGH/Karyotyper

1. Choose **New** from the File Menu and then new document window come up on the screen.
2. Select **CGH Analysis** and click **OK**.
3. Select the appropriate file where images with **ICS-format file** from IP Lab were saved.
4. By using Display Options, Tools, Image selection, and Reference, images are produced by appropriate automatic karyotyping and displayed CGH profiles automatically. Errors in karyotyping were corrected manually.
5. Select **Save As** from the file menu and save as a PICT file in allocated folder.

ii) Interpreting multiple CGH ratio profiles

1. Choose **New** from the file menu.
2. Select **CGH Interpretation** from the File Type menu on the dialog box that appears.
3. Select **the appropriate file** from the list.
4. Click **Open**.
5. Select an image analysed.
6. Click **Add** and repeat as needed to add images to the interpretation.
7. Click **Done** and then ratio profile appears on the screen.
8. Adjust the threshold to obtain accurate CGH result.

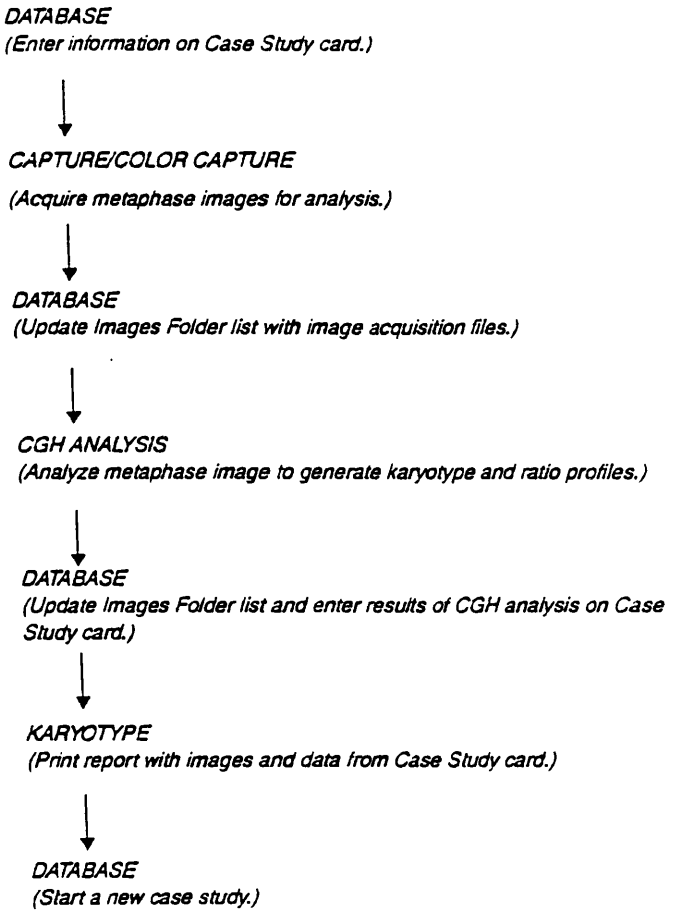


Figure 1-2. Using the Quips Suite to Analyze Case Study Data

Fig 2.7-A Diagram of the sequence using Quips system (adoped from Quips CGH analysis user guide)

Software	*Features*
Quips Karyotyping	Capture and visualize images from the microscope using a digital CCD camera or images captured using SmartCapture FISH.
	Segment chromosomes automatically, with manual correction.
	Edit metaphase and karyotype using tools and an interface that is intuitive.
	Examine selected chromosomes on a karyotype in the metaphase spread.
	Classify G-, R-, Q-, and DAPI-banded chromosomes automatically, with manual correction.
	Enhance metaphase or selected chromosomes in the karyotype.
	Generate classifiers from your metaphases appropriate for your laboratory preparations.
	Display ideograms.
	Annotate images with text, arrows, and boxes.
	Organize and print reports.
	Enter and manage case study information.
Quips CGH Analysis	Contains features of Quips Karyotyping. Use multicolored images from the microscope obtained by Quips SmartCapture FISH.
	Correct localized background automatically
	Normalize fluorescent intensities automatically.
	Enhance DAPI banding automatically.
	Display metaphase as individual color plane or composite color.
	Display karyotype as individual color plane or composite color.
	Compute chromosome symmetry axis and CGH ratio profiles automatically.
	Optionally include chromosomes in karyotype but not in profile analysis.
	Display ideograms with karyotype and ratio profiles.
	Display individual or average ratio profiles.
	Compute regions of DNA copy number gain or loss.

Table 2.7.1 Quips software system (adoped from Quips CGH analysis user guide)

Data interpretation

Data interpretation is a very important and continually developing aspect of CGH. A normal (test)-versus-normal (control) hybridization should be run with each experiment as a control. For deciding threshold, this could be fixed or could be set for each experiment by comparing normal versus normal hybridization and test vs. reference hybridization. Upper thresholds of between 1.10 and 1.25, and lower thresholds between 0.75 and 0.90, are currently accepted. In the early part of this work, these were often justified by experiment with normal versus normal hybridization. Later, limits were set at a lower threshold of about 0.8 and upper threshold of around 1.2 by comparing normal versus normal hybridization. And also statistical justification (standard deviation, 95% or 99% confidence value) was added for increasing the sensitivity of experiment. Centromeric and heterochromatic regions, as well as the p arms of acrocentric chromosomes were excluded from interpretation because regions rich in repetitive sequences show wide variability in the CGH ratio. The interpretation of the GC-rich regions at 1 pter, 16p, 19, and 22 should be carefully performed. Telomeric regions were also carefully examined because of the decreasing hybridisation signal at the very ends of chromosomes. All regions mentioned above, as well as centromeric and telomeric regions of all chromosomes, can give false ratio changes if the probe sizes or hybridisation conditions are not optimal, and careful interpretation is necessary.

Analysing method of CGH with hypotriploidy/tetraploidy

CGH analysis results only can represent the relative DNA sequence copy number, so diploid tumors cannot be differentiated from tetraploid because there is no difference in the relative fluorescence ratio along chromosomes.

When information can not be obtained from other diagnostic tools including conventional cytogenetics or FISH, CGH analysis for triploidy/ tetraplidy is impossible.

In cases with hypotriploidy/hyperdiplody or near tetraploidy, based on the

conventional karyotyping result or interphase FISH results, chromosomes with known ploidies are identified and use as the standard chromosome for CGH normalisation process. In a global analysis using the SmartCapture system, the standard chromosome which is known ploidy is normalised by equalising the fluorescence intensities to the balanced state. Then green to red ratios along the remaining chromosomes are calculated under the previous fixed normalisation state. Repeat this single chromosome normalisation method until the ploidy state of each chromosome is fully evaluated.

2.7 Whole Genome Amplification

Comparative Genomic Hybridization (CGH) of amplified DNA isolated from formalin-fixed, paraffin-embedded tissue can be difficult because of the poor quality of template DNA for amplification. In samples after the formalin fixation process, DNA is complexed with proteins and is often nicked already. The DNA concentration is too low to perform the CGH experiment. To overcome these drawbacks, universal amplification is applied to very small amounts of DNA isolated from paraffin-embedded tissue.
In this study, two kinds of whole genome amplification method were applied: primer extension pre-amplification (PEP) and degenerate oligonucleotide-primed PCR (DOP-PCR).

2.8.1 Microdissection of the specimen

7 μm and 3 μm thickness sections were prepared at Dermatology department. One of the sections was stained with hematoxylin and eosin and used as a control. The adjacent 7 μm and 3 μm sections were used for microdissection. Unstained tissue sections on glass slides were deparaffinized twice with histoclear, washed twice with 100% ethanol. Cells on the same site comparing with H-E stained slide were selected and microdissected under the inverted

microscope using 25G needle and cells were put straight into the buffer. Microdissected areas were 2mm^2 x 3μm, 9 mm^2 x 3 μm, 18 mm^2 x 3μm, 9 mm^2 x 7 μm.

2.8.2 Primer Extension Pre-amplification (PEP)

Primer

The advantage of the PEP protocol depends on random nature of primer. The primers used for PEP are a random 12 mer and a 15 mer oligonucleotides, which are made by Cruachem. Primers were already purified.
For estimation of primer concentration, 5 μl of primer was added to 995 μl of dH_2O in a 1.5-ml microcentrifuge tube, and mixed well by vortexing. Optical density of DNA was measured at A_{260} using UV spectrophotometry and the concentration was calculated as follows:

1 OD unit of a single stranded oligonucleotide consists of about 33 mg, by mass. One molar (1 M) of oligonucleotide has a number of OD units equal to 10x the number of bases.
The gram molecular weight in 12 mer oligonucleotides = 12 x 10 x 33 = 3960 g
1 M = 3960 g/L
200 pmol corresponded to 792 ng/μl
The gram molecular weight in 15 mer oligonucleotides = 15 x 10 x 33 = 4950 g
1 M = 4950 g/L
200 pmol corresponded to 990 ng/μl

PEP reaction solution

200 pmol of random 12- or 15 mer oligonucleotides
200 μM of each dNTP (Pharmacia Biotech)
5 mM $MgCl_2$ (Perkin Elmer)

1x PCR buffer 11 (Perkin Elmer)

10 µl of DNA template

5 U AmpliTaq DNA polymerase or AmpliTaq LD (Perkin-Elmer)

Add dH_2O up to 60 µl of total volume

Thermal cycler

OMN-E thermal cycler (Hybaid) was used during amplification process.

Procedure

DNA was isolated from formalin-fixed paraffin-embedded tissue sections of unknown fixation method or fixation time by the DNA isolation method No. 4, 9-2, 10, 11, 12 (Table 3.5.2).

To briefly mention again the basic method of DNA isolation, transfer samples into 20 µl of 1x PCR buffer 11 (Perkin-Elmer, Norwalk, CT, USA), heat at 95°C for 10 minutes under paraffin oil with periodic mixing to aid deparaffinization. Samples were digested with proteinase K (2mg/ml) at 65°C for 90 minutes, then the enzyme was inactivated at 85°C for 15 minutes.

In parallel experiments, 5 U of AmpliTaq LD or 3.6 U of EHF PCR system, EL Template PCR system were used as an enzyme for PEP amplification instead of AmpliTaq DNA polymerase, and the concentration of $MgCl_2$ was applied with 5 mM or 2.5 mM for comparing the results. In some experiments, primer concentration was reduced to 100 pmol. Negative control (water blanks) were included for checking contamination in each experiment.

The basic thermal cycling program (Faulkner et al.,1998) was carried out as follows:

Cycle tempreature	Time	Cycles
92°C	4 min	1 cycle
92°C 25°C 30°C 35°C 40°C 72°C	1 min 2 min 30 sec 30 sec 30 sec 2 min	40 cycles the ramp between 25°C and 30°C steps at 0.25°C/sec
72°C	15 min	1 cycle

In amplification with Pfu DNA polymerase, extension time was changed from 72°C for 2min to 72°C for 4min.

When EHF PCR system or ELT PCR system was used as an enzyme for PEP, thermal cycling program was changed as follows:

Cycle temperature	Time	Cycles
94°C 37°C 55°C 68°C	1 min 2 min 4 min 30 sec	50 cycles the ramp between 37°C and 55°C at 0.1°C/sec

2.8.3 Degenerate Oligonucleotide primer-PCR (DOP-PCR)

Primer

A degenerate oligonucleotide primer of 22 nucleotides in length with the sequence (Telenius, 1992) as follows:

5′-C C G A C T C G A G N N N N N N A T G T G G-3′

The primer was synthesized by a 391 Synthesizer PCR-MATE (Applied Biosystem). Oligonucleotide deprotection was then carried out with ethanol precipitation of primer.

Oligonucleotide deprotection

1. Connect a polypropylene syringe containing 1 ml of ammonium hydroxide (40 mM) to the end of the column (primer).
2. Attach an empty syringe to the other end.
3. Gently push the ammonium hydroxide through the column, consequently the barrel of the other syringe was displaced.
4. Push ammonium hydroxide back and forth through the column, 2 to 3 times, for 2-3 hrs. The column should be filled with liquid, which will decolourise from yellow to white.
5. Withdraw the ammonium hydroxide and transfer into small Nunc tube.
6. Add ammonium hydroxide up to 2.5 ml, tighten the cap and incubate in a 55°C water bath overnight.
7. Store at – 70°C.

Ethanol precipitation of oligonucleotides

1. Aliquot 450 µl of the deprotected primer into a 1.5 ml microcentrifuge tube.
2. Add 50 µl of 3M sodium acetate and 2 volumes of 95% ethanol and mix

gently for 30sec to 1 min.

3. Incubate the primer at –70°C for 1 1/2 hrs and centrifuge at 12000 rpm for 20 min.
4. Remove the supernatant carefully, wash the pellet with 85% ethanol and centrifuge at 12000 rpm for 2 min.
5. Discard the ethanol and allow the pellet to air dry.
6. Resuspend the pellet with 200 µl of TE buffer and store at –20°C.

Calculation method of DOP-primer concentration

For estimation of primer concentration, 5 µl of primer is added to 995 µl of dH_2O in a 1.5 ml microcentrifuge tube, mixed well by vortexing. Optical density of DNA is measured at A_{260} using UV spectrophotometry and the concentration is calculated as follows:

1 OD unit of a single stranded oligonucleotide consists of about 33 mg, by mass. One molar (1 M) of oligonucleotide has a number of OD units equal to 10x the number of bases.

The gram molecular weight in 22 oligonucleotides = 22 x 10 x 33 = 7260 g

1 M = 7260 g/L

100 pmol corresponded to 726 ng/ul

Solutions for DOP-PCR

10x PCR buffer11 (Perkin Elmer)

100 mM Tris-HCl, pH 8.3 (at 25°C)

500 mM KCl

10x EHF PCR system buffer (Boehringer Mannheim)

Enzymes:

Topoisomerase1 (1U/µl, Promega)

T7 sequenase (13 U/µl, Amersham-Life Science)

Thermosequenase (32 U/μl, Amersham-Life Science)

AmpliTaq DNA polymerase LD (5U/μl, Perkin Elmer)

Expand High Fidelity PCR System (3.5U/μl, Boehringer Mannheim)

Stock Solution

25 mM $MgCl_2$

1 M Tris-HCl

0.5 M EDTA

5 M NaCl

1 M KCl

100 μM DOP-PCR primer

2 mM each dNTP

The thermal controller-cycler

OMN-E thermal cycler (Hybaid) was used during amplification process.

Procedure

In this experiment, basically three kinds of DOP-PCR methods were used.

A) The first method by Guan et al.

First DOP-PCR amplification

1. Pipette 2 μl of PCR mix into 0.2 ml microcentrifuge tube.

   40 mM Tris-HCl (pH 7.5)

   2 mM $MgCl_2$

   50 mM NaCl

   0.2 mM dNTP

   7 pmol DOP-primer

   dH_2O

2. Add 3 μl of template DNA
3. Turn on the PCR thermal cycler and set the appropriate program
4. Add 0.1 μl of Topoisomerase 1 (1 unit) and incubate at 37°C for 30 minutes in the thermal cycler
5. Heat at 95°C for 10 minutes for inactivation of Topoisomerase 1.
6. Start 7 cycles of first low annealing temperature cycle by adding 0.2 μl (~ 0.3 units) of T7 sequenase (diluted 1:8) before starting each cycle.

    94°C for 1 min

    30°C for 1 min 30 sec

    37°C for 2 min x 7

7. After finishing 7 cycles, add 45 μl of PCR mixture and mix well.

    10 mM Tris-HCl

    50 mM KCl

    2 mM $MgCl_2$

    75 pmol DOP-primer

    0.2mM dNTP

    dH_2O

8. Heat at 95°C for 3 minutes as a denaturation step in the thermal cycler.
9. Pause the thermal cycler.
10. Add 0.8 μl of AmpliTaq LD.
11. Start again first high annealing temperature cycles.

    94°C for 1 min

    56°C for 1 min

    72°C for 3 min x 35

    72°C for 10 min x 1

Second DOP-PCR amplification

1. Add 7 μl of first DOP-PCR product into the second PCR mixture.

   1x PCR buffer 11

   2.5 mM $MgCl_2$

   0.2 mM dNTP

   150 pmol DOP-primer

   dH_2O (up to 50 μl)

2. Denature at 95°C for 3 minutes in the thermal cycler
3. Add 0.7-0.8 μl of AmpliTaq LD in to the mixture
4. Start the second DOP-PCR thermal cycles

   94°C for 1 min

   56°C for 1 min

   72°C for 3 min x 30

   72°C for 15 min x 1

B) The second method by Kuukasjärvi et al. (1997)

First DOP-PCR amplification

1. Add 3 μl of template DNA into the 2 μl of the PCR mixture prepared (total volume; 5 μl).

   20 mM Tris-HCl

   60 mM KCl

   2 mM $MgCl_2$

   0.2 mM dNTP

   5 pmol DOP-primer

   0.5 ul of thermosequenase

   dH_2O

2. Start the first low annealing temperature cycles in thermal cycler.

   94°C for 1 min

   25°C for 1 min with 3 min ramp from 25°C to 74°C

   74°C for 2 min x 4

3. After finishing 4 cycles, add 20 µl of PCR mixture into the previous product.

   10 mM Tris-HCl (pH 8.4)

   10 mM KCl

   1.5mM $MgCl_2$

   0.2 mM dNTP

   50 pmol DOP-primer

   0.5 µl of AmpliTaq LD

   dH_2O

4. Start the first high annealing temperature cycles in the thermal cycler.

   94°C for 1min

   56°C for 1 min

   72°C for 2 min x 30

Second DOP-PCR amplification

Repeat the above step 3 and 4.

(template DNA ; product from first high temperature cycles)

C) The third method

First DOP-PCR amplification

1. Prepare 49.2 µl of PCR mixture in the 0.2 ml microcentrifuge tube.

   1x PCR buffer 11

   1.5mM $MgCl_2$

   0.2 mM dNTP

   100 pmol DOP-primer

   10 µl of template DNA

   dH2O

2. Denaturate the PCR mixture at 96°C for 8 minutes.
3. Add 0.8 µl of AmpliTaq LD.

4. Start the first low annealing temperature cycles in thermal cycler.

   93°C for 1 min

   30°C for 1 min with 3 min ramp from 30°C to 72°C

   72°C for 3 min x 8

5. Continue the high annealing temperature cycles without interruption.

   93°C for 1 min

   60°C for 1 min

   72°C for 3 min x 28

   72°C for 15 min x 1

Second DOP-PCR amplification

1. Prepare 50 µl of second DOP-PCR mixture in the 0.2 ml microcentrifuge tube

   1x PCR buffer 11

   2.5 mM $MgCl_2$

   0.2 mM dNTP

   150 pmol DOP-primer

   1µl of AmpliTaq LD

   10 µl of first DOP-PCR product as a template DNA

   dH_2O

2. Start the thermal cycles in the thermal cycler.

   93°C for 1 min

   60°C for 1 min

   72°C for 3 min x 28

   72°C for 15 min x 1

2.8.4 Gel electrophoresis of the amplification product

The length of the probe molecules can be checked by gel electrophoresis.

1. Weigh out 0.6 g of agarose and added to 60 µl of TAE or TBE buffer.
2. Dissolve it by boiling in the microwave oven for 3 minutes.
3. Allow to cool down to 70°C, and add 2 µl of ethidium bromide to the dissolved agarose solution inside the hume hood.
4. Pour the gel prepared (1 %) onto the electrophoresis gel casting unit.
5. Mix 4 µl of probe with 1 µl of loading mix and load on the gel.
6. Run the gel at 15V/cm for 30 minutes and take photographs under ultraviolet illumination.

The amount of loading mix and probe can be changed depending on size of well. Every time, ladder is loaded on the gel.

2.8.5 Measurement of probe concentration

The concentration of the DNA probe is estimated by measuring the optical density (O.D.) at A260 nm in a spectrophotometer. 5 µl of the probe DNA is added to 995 µl of dH2O, mixed well by vortexing and then measured. The concentration of the probe is calculated according to the formula below:

Conc = OD A260 x dilution factor (200) x 50 = concentration of probe µg/ml or ng/µl

A260: Absorbance at 260 nm wavelength

50: a Constant corresponding to 50 µg DNA per ml at 260 nm per 1 unit OD

2.8.6 Probe labelling with amplified probe

There are two labelling methods after amplification namely DOP-PCR labelling and nick translation.

In this study, products of PEP amplification were usually labelled by nick translation, products of DOP-PCR amplification were labelled by DOP-PCR labelling.

DOP-PCR labelling

During the second DOP-PCR amplification, fluorochrome (5 μl of FITC-12-dUTP for test DNA and 5 μl of TexasRed-5-dUTP for control DNA; Du Pont or 2.5 μl of FITC-11-dUTP for test DNA and 2.5 μl of rhodamine-4-dUTP; Amersham) was put into PCR reaction mixture for labelling.
After finishing second DOP-PCR amplification, labelled products were subject to a purification step to remove any unincorporated nucleotides. But CGH results with amplified product using DOP-PCR were rarely affected by purification.

Nick translation

Same as labelling method in CGH experiment

2.8.7 Purification of labelled probe

At first, purification of labelled probe was carried out using High Pure PCR Product Purification Kit.

High Pure PCR Product Purification Kit (Boehringer Mannheim)

Materials

Binding buffer

3 M guanidine-thiocyanate
10 mM Tris-HCl
5% ethanol (v/v), pH 6.6 (25°C)

Wash buffer

20 mM NaCl

2 mM Tris-HCl, pH 7.5 (25C)

Elution buffer

10 mM Tris-HCl

1 mM EDTA, pH 8.5 (25C)

High Pure filter tube

Collection tube

Procedure:

1. Add 500 µl binding buffer to a 100 µl PCR reaction and mix well.
2. Combine the High Pure filter tube and the collection tube and transfer sample to the upper reservoir.
3. Centrifuge for 30 seconds at maximum speed in a standard table top centrifuge.
4. Discard the flow through and combine the filter tube again with the same collection tube.
5. Add 500 µl wash buffer to the upper reservoir and centrifuge for 30 sec at maximum speed.
6. Discard the wash buffer flow through and combine the filter tube again with the same collection tube. Add 200 µl wash buffer, centrifuge as in step 3.
7. Discard the collection tube and insert the filter tube in a clean 1.5 ml reaction tube.
8. Use 50-100 µl elution buffer or redistilled water for the elution of DNA. Add elution buffer or water to the filter tube and centrifuge at maximum speed for 30 sec.

2.9 PCR with specific primers

PCR for sex determination; a 30 mer derived from pseudoautosomal sequences (C) in combination with 30 mer from Y-specific and X-specific regions

Sequence of primers

X CTGCAGAAACAAGCTCATCAGCGTGACTAT

Y GTACTACCTTTAGAAAACTAGTATTTTCCC

C GAATTCTTAACAGGACCCATTTAGGATTAA

The thermal controller-cycler

OMN-E thermal cycler (Hybaid) was used during amplification process.

Procedure

1. Prepare the primer mix according to C:Y:X at a ratio of 50:75:5 using 10 μM stocks of each primer.
2. Prepare the PCR beads (two in one tube: Amersham Pharmacia biotech) and add 25 μl of distilled water, 2 μl of template DNA (100 ng), and 23 μl of primer mix.
3. Start the thermal cycles in the thermal cycler

94°C for 5 min
54°C for 1 min
72°C for 2 min x 1 cycle

94°C for 1 min
54°C for 1 min
72°C for 2 min x 30 cycles

72°C for 5 min x 1 cycle

CHAPTER 3. RESULTS

3. RESULTS

3.1 Fluorescence In Situ Hybridisation

In order to become familiar with the techniques of Fluorescence In Situ Hybridisation (FISH), a number of control experiments were carried out using libraries to whole chromosomes (including suppression), centromeric probes to chromosomes and centromeric probes to nuclei.

3.1.1 Interphase Fluorescence In Situ Hybridisation

Conventional G-banding analysis of childhood ALL is technically more difficult than in other leukemias. For detection of structural aberrations or numerical abnormalities, high quality chromosome preparations from bone marrow are required. But malignant clones of the B-cell lineage can be obtained only by rapid harvesting because of rapidly decreasing malignant cells during prolonged in vitro culture (Karhu et al., 1997b). In practice, between 23 and 33% of cases cannot produce adequate or sufficient metaphases to identify hyperdiploidy (Jackson et al., 1990b; Harris et al., 1992; Moorman et al., 1996).
Moorman et al. (1996) indicated the potential of interphase FISH in this situation and recommended a sequence of probes based on the involvement of specific chromosomes in hyperdiploidy (Fig 3.1-A).
The first objective of this project was to try to detect hyperdiploidy when conventional karyotyping had failed, or hidden hyperdiploidy in ALL patients using interphase FISH with Moorman's strategy. The second aim of this experiment was to classify the cases studied into one of the three ploidy subgroups; low hyperdiploidy with 47-50 chromosomes, high hyperdiploidy with 51-68 chromosomes and tripoidy/tetraploidy with >68 chromosomes.
This study was carried out on cells which had been cultured for diagnostic chromosome analysis. The probes were selected specific chromosome alpha-satellite probes, in a step-wise pattern according to Moorman's strategy, for chromosomes known to be frequently involved in hyperdiploidy (Fig. 3.1-A).

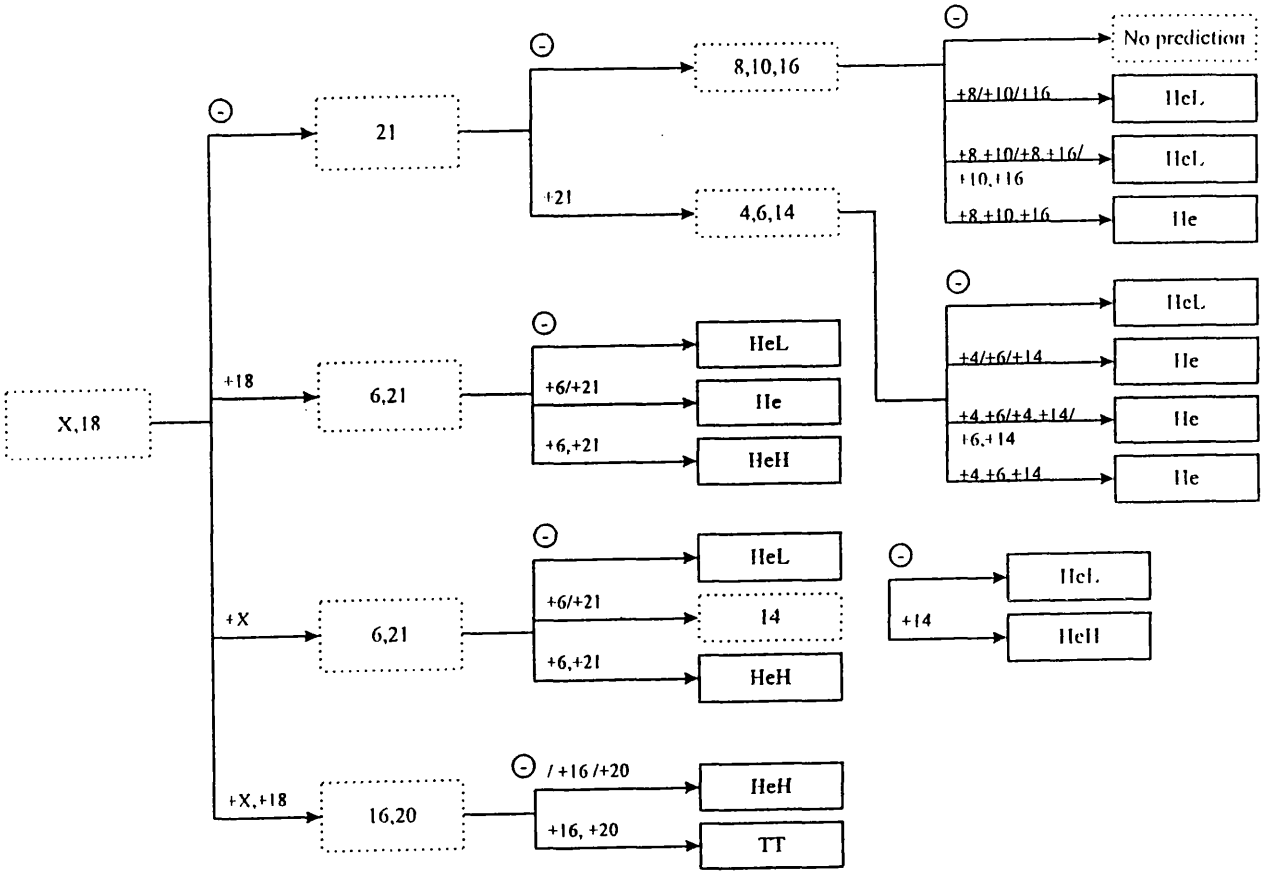


Fig 3.1-A Probes for detection of hyperdiploidy in ALL according to Moorman et al. (adopted from Moorman et al, Genes, Chromosomes & Cancer 16:40-45, 1996).

For the 22 samples studied, conventional karyotyping and interphase FISH were carried out independently.

At least 100 interphase nuclei were analysed for each probe on each sample. Every experiment was carried out in parallel with a control sample and the results of test and normal control samples were compared. When the signals were located close together with the same intensity and of similar size, they were scored as one signal. When size and intensity of signals were different and there was more one signal space between the two signals, they were treated as two signals.

Complete karyotype and intephase FISH results of the 22 cases are listed in Table 3.1.1.

Five cases had failed to yield suitable material for cytogenetic analysis. In three of these cases (numbers 5, 12, and 17), no abnormality was detected by interphase FISH for the probes used. In the remaining 2 cases, case 9 was shown to have additional copies of chromosome 4 and 20 in a proportion of cells (3 signals; 7% in normal control) and also to lack either a chromosome 13 or 21 in over 60% of cells (3 signals; 17%, 4 signals; 74% in normal control), but it was difficult to identify the exact hyperdiploid group. Case 20 was shown to have an additional copy of chromosome 18 and chromosome 6 in the majority of cells, and can be allocated into the hyperdiploid group which are according to the strategy of this experiment (Fig 3.1-A).

Six of the 22 cases studied had a normal karyotype by conventional cytogenetic analysis (cases 8, 11, 14, 15, 16, and 18). No abnormalities were detected by the probes used.

Three cases were identified by conventional cytogenetics as having structural abnormalities (cases 1, 4, and 10) and again no further abnormality was detected by the probes used here.

Three further cases (cases 7, 19, and 22) had shown both structural and numerical abnormalities on G-banding. In case 7, no abnormality was detected by the panel of probes, while in case 19 the presence of an additional chromosome 8 was confirmed (3 signals; 2 % in normal control). However in case 22 interphase FISH using probes for four chromosomes including chromosome 8 did not confirm the trisomy 8 detected by cytogenetic analysis. This was thought to be caused by mosaicism with the additional abnormal clone and normal cells.

Four hyperdiploid cases had been identified by conventional cytogenetics of which three (cases 2, 13,and 21) were in the high hyperdiploidy subgroup

and one (case 6) was in the low hyperdiploidy subgroup. In case 2 the cytogenetic analysis had been incomplete, and FISH allowed identification of three chromosomes involved as chromosome X, 6, and 13 or 21. In cases 6, 13, and 21, FISH confirmed the cytogenetic results where probes for the chromosomes involved were included in the panel.

The final case (case 3) was in the triploid/tetraploid subgroup having been shown to have a count of 90 chromosomes and almost complete tetraploidy. FISH for the probes used was in almost complete agreement with this result, although it was interesting that 66% of nuclei showed 4 signals for chromosome 12, which was one of the two chromosomes with trisomy by conventional analysis (2 signals; 91%, 3 signals; 2% in normal control).

In some cases, the percentage of abnormal signals was not definite but was significantly different from the normal control. For example, in case 13, the percentage of abnormal signals of chromosome 18 (3 signals) was 44% compared with below 5% in normal control. This was thought to be caused by mosaicism with normal cells.

All results of interphase FISH for the probes used were consistent with the results from conventional karyotyping and in addition FISH provided information in the cases which could not be analysed by routine cytogenetics.

Table 3.1.1 Summary of the results of interphase FISH & conventional karyotyping of 22 cases with ALL

No	Karyotype	Probes	Number of signals per nucleus (%)					
			0	1	2	3	4	5
1	46,XY,i(9)(q10), del(13)(q12q22), der(19)t(1;19)(q23;p13)	X		85	15			
		18		2	96		2	
		13/21	3	2	10	18	60	7
		10		19	72	9		
		16		9	79	11		
2	50-53,XY	X		5	94*	1		
		18	2	10	78	10		
		6	1	2	28	67*	2	
		13/21		2	2	10	53	33*
3	90,XX,YY,del(2)(p?)x2,-4, t(8;14)(q24;q32)x2,add(11)(q23)x2, -12,del(17)(p11)x2	X			83*	13	4	
		18			12	19	67*	2
		16			7	9	77*	7
		20			6	11	74*	9
		12			2	32*	66*	
4	46,XX,del(6),del(12),add(12)	X	2	11	87			
		6		5	95			
		8		5	86	9		
		10		10	82	8		
5	46,XX (failed case)	X		8	89	3		
		6		9	91			
		8		5	94	1		
		10		12	86	2		
6	49-50,XX,del(5),+6,-11,+12,+17,-22	X			84	16		
		6		4	19	75*	2	
		8		3	87	4	3	3
		10			89	10	1	
7	45,XX,add(1)(q?21),add(2)(q14), der(3)t(1;3)(q21;p25),del(6)(q21), -7,der(9)add(9)(p12),add(9)(q34), del(22)(q11), 45,idem,del(11)(q23) 46,XX	X		5	94	1		
		18		10	85	5		
		13/21			3	11	82	4
		10		8	90	2		
		16		1	88	11		
		8		1	88	9		
8	46,XY	X	2	96	2			
		18		4	92	4		
		13/21			3	11	83	3
		10		6	89	5		
		16		6	86	7	1	
		8		7	87	5	1	
9	Failed case	X		5	91	4		
		18		7	91	2		
		13/21			7	66*	27	
		10		10	87	3		
		16		12	85	3		
		4			5	58*	37*	
		6		5	95			
		8		6	94			
		20		2	78	20*		

No	Karyotype	Probes	Number of signals per nucleus					
			0	1	2	3	4	5
10	46,XX,t(1;13)(p10;q10) 46,XX	X		1	94	5		
		18	1	6	93			
		13/21			9	18	72	1
		10		10	87	3		
		16		7	88	5		
		8		8	86	6		
11	46,XX	X		1	93	6		
		18		10	90			
		13/21			3	17	79	1
		10		10	90			
		16		8	88	4		
		8		11	86	3		
12	Failed case	X		89	10	1		
		18		4	90	6		
		13/21			6	18	75	1
		10		4	92	4		
		16		3	88	9		
		8		6	92	2		
13	53,XY,+X,+6,+10,+14,+18,+21,+21 46,XY	X		3	87*	10		
		18		3	52	44*		
		13/21				1	9	83*
		6			13	87*		
		8		8	92			
14	46,XY	X	2	95	3			
		18		7	92	1		
		13/21			3	21	76	
		10		8	92			
		16		8	90	2		
		8		11	89			
15	46,XX	X		5	93	2		
		18		4	95	1		
		13/21			3	14	81	2
		10		9	90	1		
		16		8	91	1		
		8		5	93	2		
16	46,XY	X		97	3			
		18		8	92			
		13/21			4	19	76	
		10		7	93			
		16		11	89			
		8	1	15	83	1		
17	Failed case	X		97	3			
		18		11	88	1		
		13/21			9	27	64	
		10		6	94			
		16		14	86			
		8		9	91			

No	Karyotype	Probe	Number of signals per nucleus (%)					
			0	1	2	3	4	5
18	46,XX	X		3	96	1		
		18		5	95			
		13/21			3	14	81	2
		10		11	89			
		16		8	92			
		8		2	97	1		
		6		1	96	3		
		20		8	86	6		
19	46,XX 46,XX,+8,-14, der(16)t(16;17)(q10;q10)	X		10	89	1		
		18		5	94	1		
		13/21			7	19	74	
		10		5	91	3	1	
		16		8	87	5		
		8		4	65	31*		
20	Failed case	X		15	85			
		18			34	66*		
		13/21			1	17	79	
		6			31	69*		
21	56,XY,+X,+4,+6,+8,+10,+11? +14,+18,+21,+21	X		9	91			
		18		2	66	32*		
		13/21					14	74*
		6			37	63*		
22	45,XX,-5,-22,+mar 42-47,XX,+8,-14 dic(16;17)(p11.2;p11.2), add(19)(q13?), +mar1, +mar2	X		3	97			
		18		15	84	1		
		13/21			3	19	78	
		8		5	86	9		

* abnormal result compared with normal control

3.2 Establishment of the CGH Technique

In the second part of the project, the aim was to apply CGH to the study of hematologic malignancies and to compare the results with those of conventional cytogenetic analysis. In order to become familiar with the technique, a number of control samples of both fresh and fixed bone marrow cells were processed.

Both direct and indirect labelling of test and control DNA were investigated and experience was gained in monitoring the critical factors such as metaphase preparations, probe size, and hybridisation conditions.

Initially, CGH experiments were carried out according to the indirect method as described by Kallioniemi et al (1992) and Du Manoir et al (1993). The results obtained were unsatisfactory because the intensity of control DNA (red) was weaker than that of the test DNA (green). After several experiments with modifications, it was possible to obtain analyzable CGH results (Fig 3.2-A). The direct labelling method by Kallioniemi et al (1994) was then applied to CGH experiments with satisfactory results (Fig 3.2-B). The trial group was too small to compare both methods exactly. From literature reviews, it appears that CGH with directly labelled probe can produce more uniformly hybridized images and fewer non-specific signals and is more convenient to use than that with indirectly labelled probe (Buckle and Kearney, 1994; Isola et al., 1994).

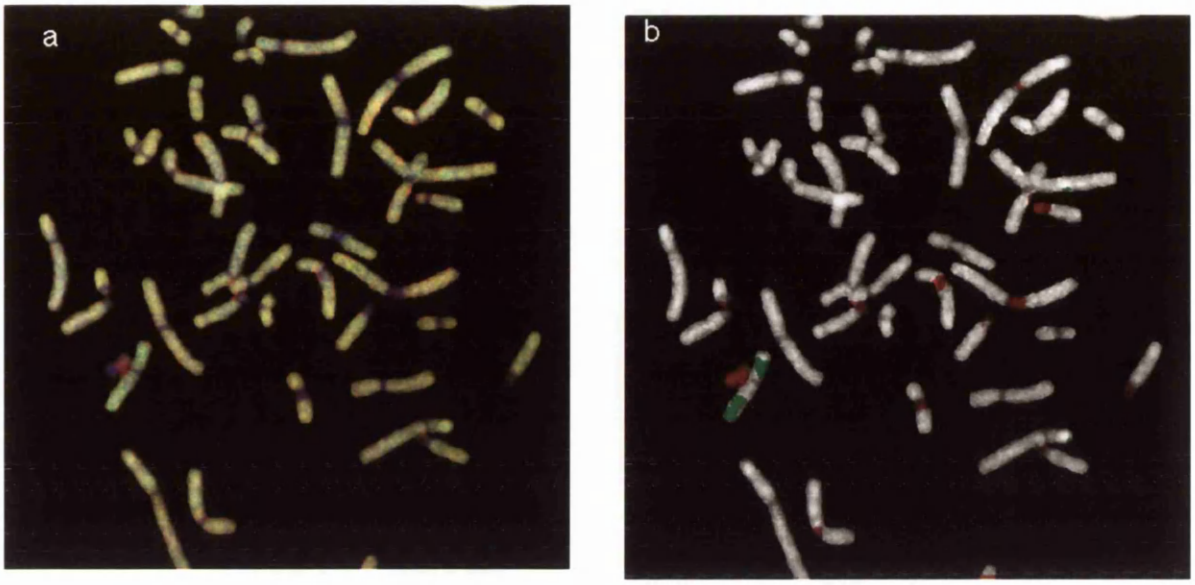


FIG 3.2-A Global CGH image using indirect labelling method; test DNA was normal female DNA labelled with Biotin - 11- dUTP, control DNA was male DNA labelled with digoxigenin -11- dUTP, and hybridised on male metaphase chromosomes. a) CGH image following colour equalisation b) colour ratio image of the same metaphase.

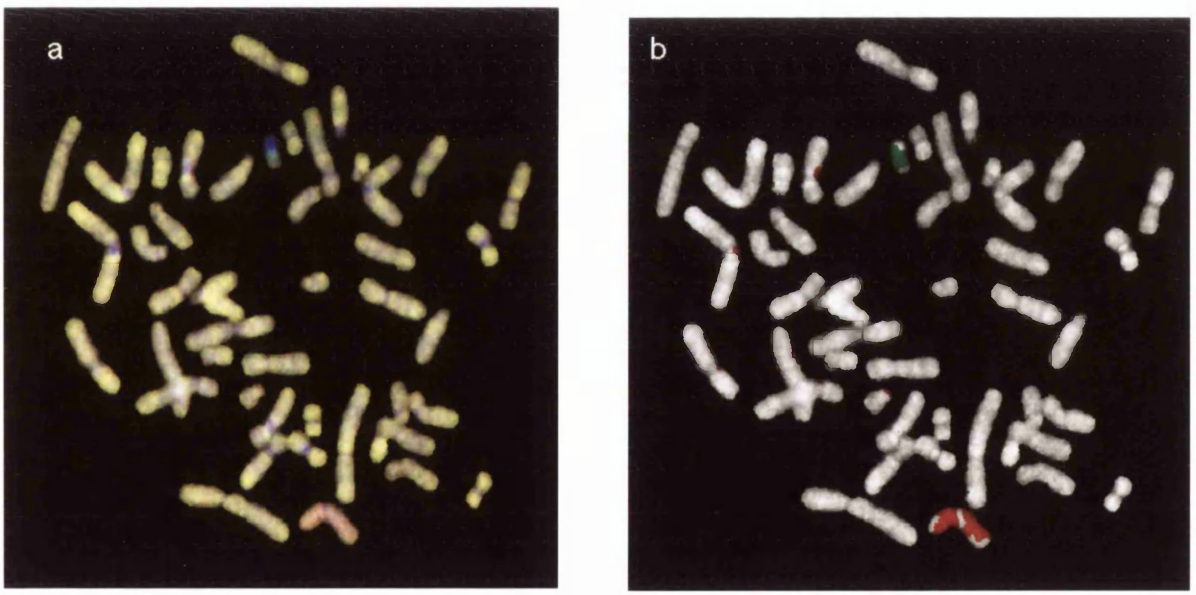


FIG 3.2-B Global CGH image using direct labelling method; test DNA was normal male labelled with FITC-12-dUTP, control DNA was female DNA labelled with Texas Red-5- dUTP, and hybridised on male metaphase chromosomes. a) CGH image following colour equalisation b) colour ratio image of the same metaphase.

3.3 CGH analysis in cases with Hematologic Malignancies

3.3.1 CGH Analysis on Samples with ALL

The aim of this study was to assess whether CGH would be an approach to help identify hyperdiploidy in cases of ALL, especially when conventional karyotyping cannot be completed due to inadequate mitoses. Interphase FISH using selected probes was also performed as a complementary method to detect hyperdiploidy in these patients.

Nineteen bone marrow samples from suspected or confirmed cases of ALL were analysed. All samples were selected randomly when sufficient material remained after setting up diagnostic cultures. In 16 cases genomic DNA was prepared from fresh bone marrow samples and in 3 cases from stored fixed metaphase preparations.

Of the 19 cases of ALL studied, 9 cases showed a normal karyotype and a normal CGH result. The results of the remaining 10 cases are shown as Table 3.3.1. The summary of interphase FISH results of three hyperdiploid/hypotriploid cases is presented in Table 3.3.2.

Three of 19 cases studied (cases 8, 14, and 19) had numerical abnormalities with or without marker chromosomes by conventional cytogenetics. CGH profiles of two hyperdiploid (case 8 & 14) are shown in Fig.3.3.1-A. Both cases were hybridised on metaphases of the same sex with test materials for detection of abnormalities on sex chromosomes.

In case 8, the CGH result was in almost complete agreement with conventional karyotyping except that gains of DNA material of chromosome X and Y were detected. FISH results using whole chromosome Y painting probe revealed that one of the marker chromosomes originated from chromosome Y material as shown in Fig 3.3.1-B. Insufficient material remained for FISH with an chromosome X library. Case 14 also showed hyperdiploidy with complete agreement with both results. Four copies of chromosome X and 21 were revealed by single chromosome normalisation of chromosome 14 which was an already known trisomy. In case 19 there was discordance between CGH and conventional karyotyping results. CGH could not detect the presence of trisomy 4 because this cell line was present in only 33% of cells by cytogenetic analysis.

Three of 19 cases (cases 13, 18, and 20) studied had shown both numerical and

structural abnormalities or structural abnormalities only on G-banding. In case 13, conventional karyotyping had shown del(6)(q21), der(9)?t(9;21)(p13;q11.2), -21, compared with CGH results which revealed loss of chromosomal regions 6q11-q21, 9p13.2-pter, and normal result for chromosome 21. Considering both results, it is likely that chromosome 21 material was rearranged to the deleted chromosome 9p and the der(9) was presumably dicentric. There was discordance between conventional karyotyping and CGH results about the breakpoint on chromosome 6. Case 18 showed high level of gain (amplification) of chromosome 6p23-6pter by CGH (Fig 3.3.1-C). The finding of add(6)(p2?) by conventional karyotyping was thought to be material from 6p23-6pter. In Case 20 cytogenetic analysis has shown loss of chromosome 4, gain of chromosome 8, and various complex chromosomal aberrations which could not be detected by CGH. CGH revealed only gain of chromosome 8 and normal result for chromosome 4. FISH using chromosome 4 library was then applied to metaphase preparations from bone marrow, and showed that chromosome 4 material was present in several rearranged chromosomes such as der(11), add(17), and ring chromosome.

The final four cases (cases 7, 9, 10, and 17) had failed to yield successful G-banding results. Two cases (cases 9 and 10) showed normal CGH result. In the remaining two cases, case 7 was shown to have loss of material chromosome Xpter-q24 but the result could not be confirmed by FISH because adequate metaphases were not available. Case 17 was shown be triploid by using CGH analysis (2-4 copies of each chromosome except chromosome X). The initial analysis (CGH) appeared to show loss of chromosome X, partial loss of chromosome 3, 9, and 12 (degree of loss of chromosome; 3> 9> 12), gains of chromosome 20 and 21, and partial gains of 16, 17, 19, and 22, and balanced state of remaining chromosomes in images by global analysis. But interphase FISH had predominantly shown 3 signals for all probes tested except chromosome X. Because CGH can only evaluate relative copy numbers, it cannot completely evaluate ploidy levels or absolute copy numbers of chromosomal regions. There are some documented limitations in detecting hyperdiploidy by existing methods of normalization. So in this case the full status of numerical abnormalities could be evaluated by global method (pixel by pixel fluorescence intensity ratio image) with multiple single chromosome normalization (Rosenberg et al., 1997) (Fig 3.3.1-D).

Interphase FISH results (cases 8, 14, and 17) corresponded with CGH results, but

case 8 showed one discrepancy (chromosome X) with conventional karyotype results (Table 3.3.2). It was thought be that chromosome X was in one of the marker chromosome.

Table 3.3.1. Results of karyotype and CGH analysis in 10 cases with ALL

Case	Sex	Karyotyype	CGH result
7	F	Fail	- X(pter-q24)
8	M	55-56,XY,+4,+6,+10,+14,+17,+18,+21, +mar1,+mar2, +mar3[CP9] 46,XY[4]	+X, +Y, +4, +6, +10, +14, +17, +18,+21
9	F	Fail	Normal
10	F	Fail	Normal
13	M	45,XY,del(6)(q21),der(9)?t(9;21)(p13;q11.2),-21	-6(q11-q21), -9(p13.2-pter)
14	F	55,XX,+X,+?X,+4,+5,+10,+14,+18,+21,+21[CP3] 46,XY[10]	+X, +X, +4, +5, +10, +14, +18, +21,+21
17	F	Fail	Normal X, +3x2/3, +9x2/3, +12x2/3(3), +16x3/4, +17x3/4, +19x3/4, +22x3/4, +20x4, +21x4, others x3
18	F	46,XX,add(6)(p2?)[CP9] 46,XX[5]	Amplification of 6(p23-pter)
19	M	47,XY,+4[CP4] 46,XY[8]	Normal
20	F	47,XX,add(3)(p25),-4,+8,inv(9)(p12q13)c, del(10)(q24),der(11)t(4;11)(q21;q23), add(17)(11.2), +r [CP8] 46,XX,inv(9)(p12q13)[2]	+8

Table 3.3.2. Interphase FISH[a] results in 3 hyperdiploid / hypotriploid cases with ALL

Ch.	Signal No / Case No	0	1	2	3	4	5	6	7	8
X	8		10%	87%*	3%					
	14			11%	13%	72%*	4%			
	17		4%	87%	8%	1%				
	C M F		99% 3%	1% 96%	 1%					
3	17			42%*	57%*	1%				
	C		2%	97%	1%					
4	8			8%	77%*	15%				
	14			22%	77%*	1%				
	17			13%	83%*	4%				
	C		3%	93%	4%					
10	8			16%	84%*					
	14			11%	81%*	7%				
	17			3%	90%*	7%*				
	C		1%	98%	1%					
12	17			12%	68%*	20%				
	C		2%	98%						
14/22	8				6%	12%	82%*			
	17							46%*	8%*	44%*
	C			8%	8%	83%	1%			
17	8			14%	83%*	3%				
	C		2%	96%	2%					
18	8			14%	84%*	2%				
	14			9%	91%*					
	C		4%	94%	2%					
21	8		2%	18%	79%*	1%				
	14			16%	10%	74%*				
	17			6%	8%	82%*	4%			
	C		8%	91%	1%					

[a] FISH, Flurorescence In Situ Hybridisation; ALL, Acute Lymphoblastic Leukemia

* abnormal result comparing with normal control

c normal control

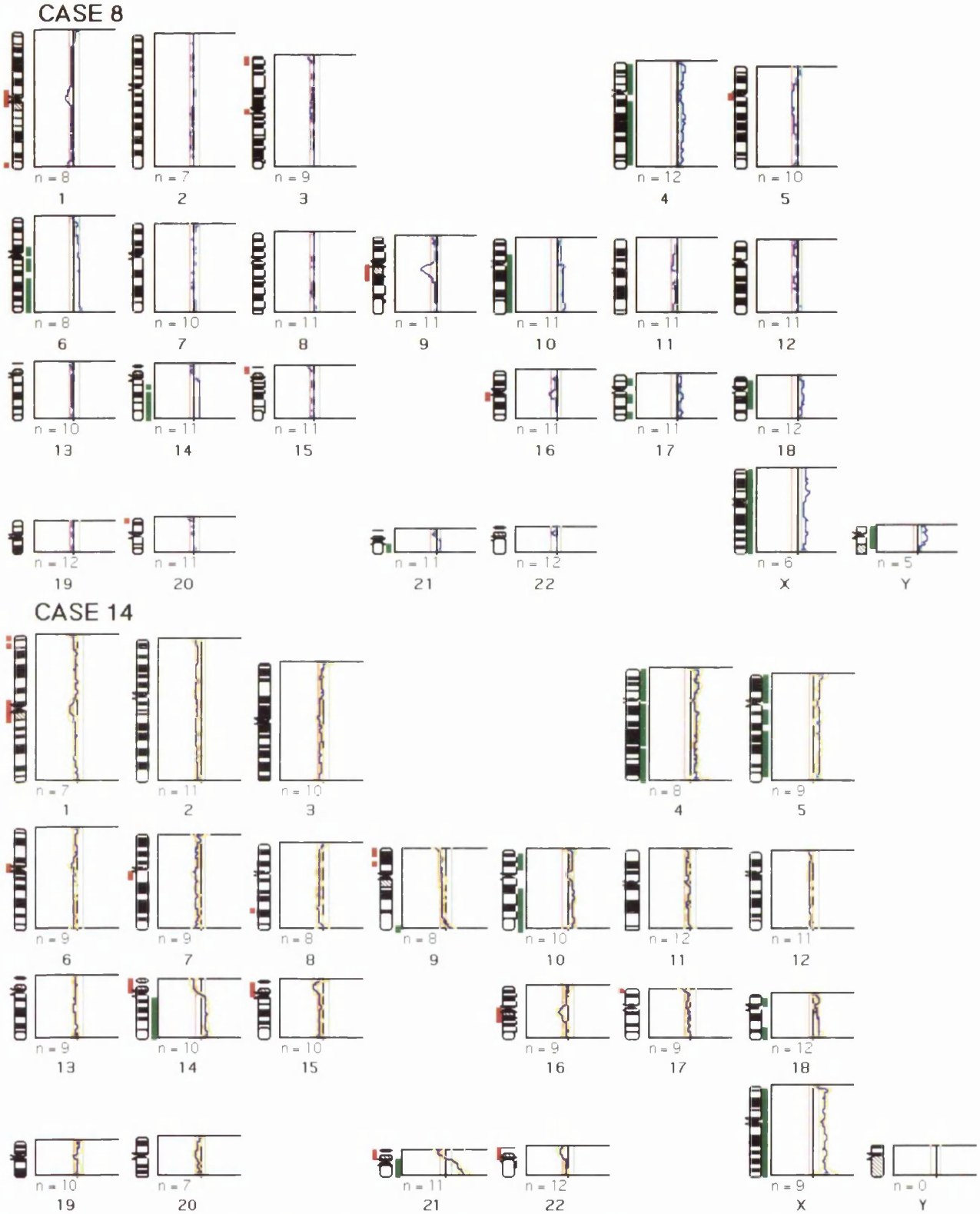


FIG 3.3.1-A CGH ratio profiles of case 8 & 14; Case 8 shows gains of material on chromosome 4, 6, 10, 14, 17, 18, 21, X, and Y and case 14 shows gains of material on chromosome 4, 5, 10, 14, 18, 21, and X. Both cases show incomplete coverage of whole length of chromosome in some chromosomes.

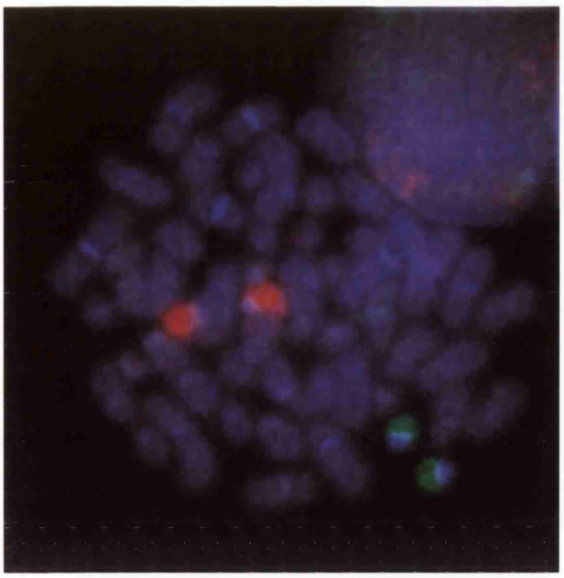


FIG 3.3.1-B Dual-colour FISH hybridized on patient metaphases (case 8) Probes used were WCP Y (green)/ chromosome 15 (red). Choromosome Y material was incorporated into one of the marker chromosomes.

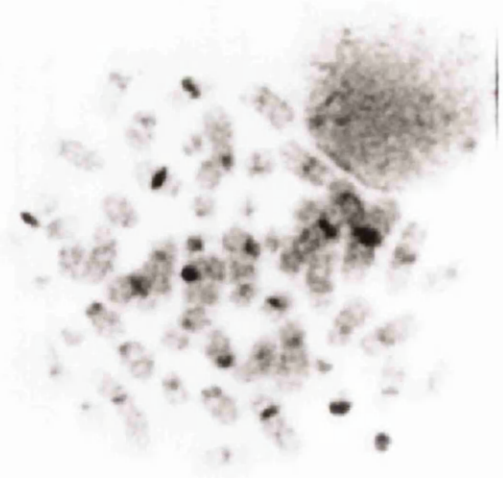


FIG 3.3.1-B black and white image of the same metaphase

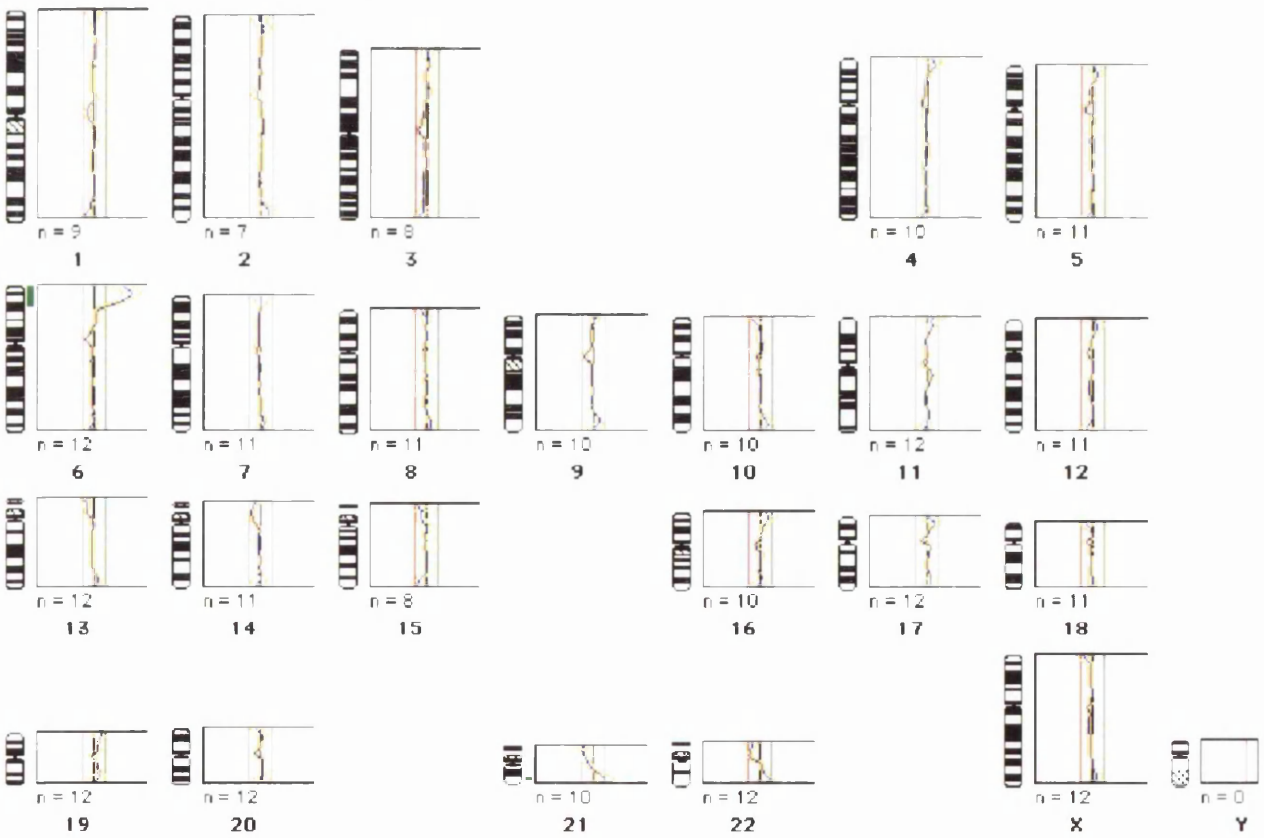
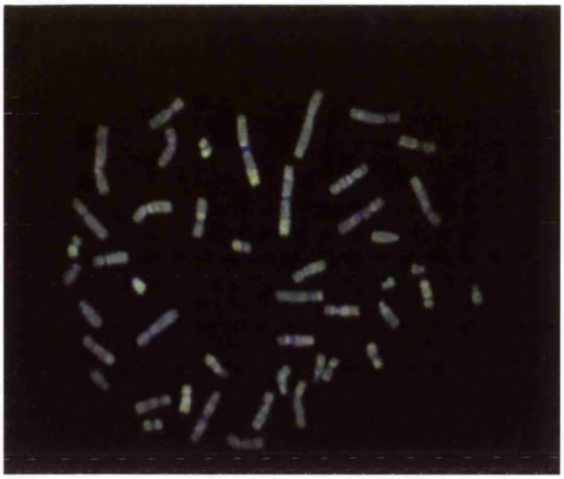
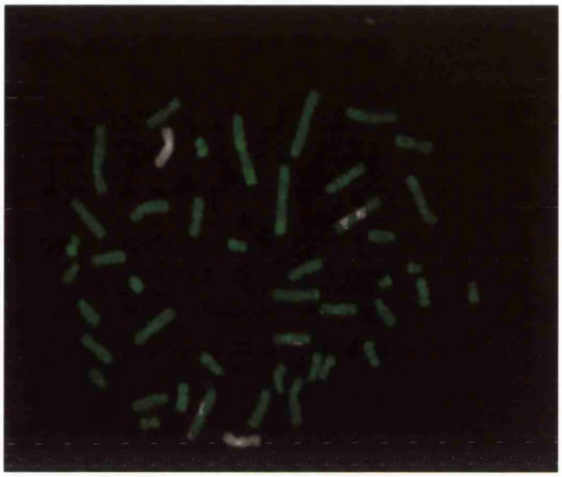


FIG 3.3.1-C CGH ratio profile of case 18, which is shown amplification on the region of chromosome 6p23-6pter

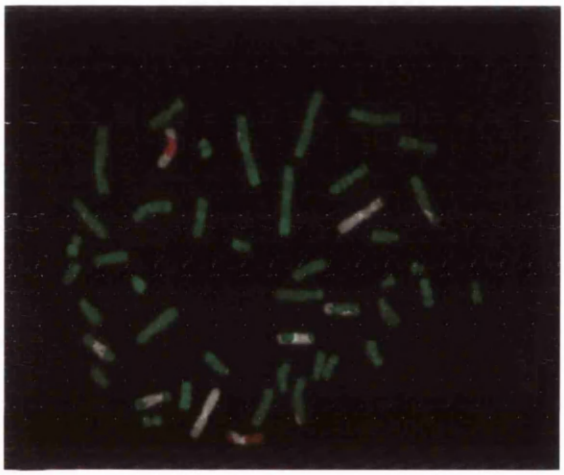
(A)



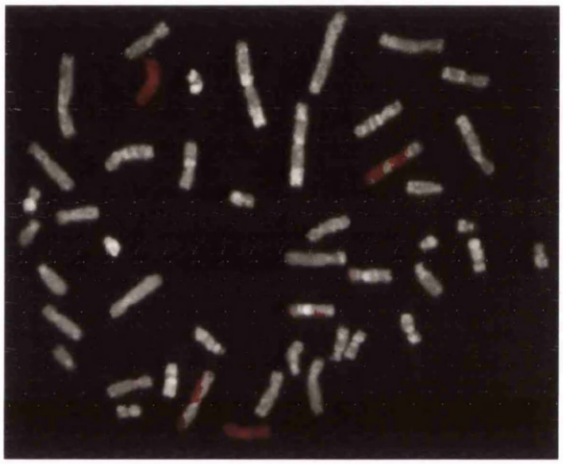
(B)



(C)



(D)



(E)

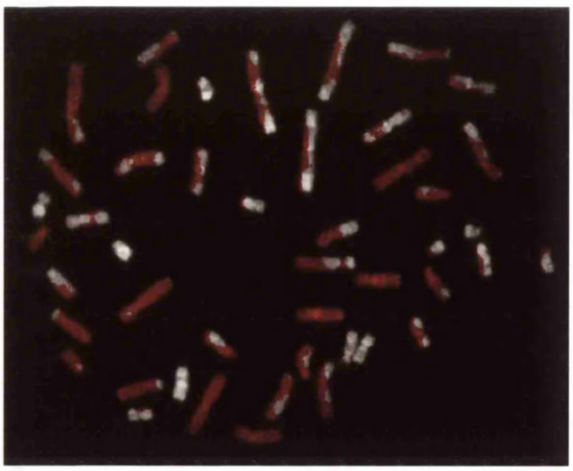


Fig 3.3.1- D: CGH image by global analysis and images following single chromosome normalisation of the same metaphase (case 17). Green; over-represented chromosome regions (FR>1), White; balanced state of the chromosome material (FR=1), RED; under-represented state of chromosome regions (FR<1). (A) CGH image without fluorescence equalisation, (B) CGH image with ch.X normalisation, (C) CGH image with ch.3 normalisation, (D) CGH image with ch.1 normalisation, (E) CGH image with ch.20 normalisation.

3.3.2 CGH Analysis in cases with AML or AML/MDS

Detection of the Origin of Marker Chromosomes by using CGH and FISH

In Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS), there are frequently karyotypes with multiple structurally altered chromosomes, many of which are marker chromosomes of unknown origin.
The aim of this study was to assess if CGH could be used as a tool to identify the origin of marker chromosomes or other unbalanced structural aberrations detected during diagnostic cytogenetic analysis.
Nineteen bone marrow samples from patients with AML or MDS in transformation were randomly selected depending on availability of the samples.
Conventional karyotyping and CGH were performed independently. FISH was performed using whole chromosome paints which were carefully chosen on the basis of discordance between CGH results and standard cytogenetic analysis.

Conventional karyotyping and CGH results

In 7 of the 19 patients, normal results were observed both by cytogenetics and CGH. Cytogenetic analysis of the remaining cases revealed complex karyotypes with multiple chromosome aberrations in 8 cases and two cases showed a balanced t(15:17); two cases failed to yield a result. CGH analysis of these two failed cases (case 8 and 17) showed a normal result in case 8 and showed multiple gains and losses of specific chromosome regions in case 17. CGH could not detect any imbalance in the two cases with balanced translocations. This is a known limitation of the technique. Complete karyotypes and CGH data of these 19 cases are listed in Table 3.3.3. A summary of 9 abnormal CGH profiles is shown in Fig.3.3.2-A.
From this data, the most frequently observed abnormality on CGH analysis is loss of material from 5q (6 out of 19 cases) especially loss of 5q23-31 (5 cases).
Cases 4 and 7 showed amplification at 8q24 (C-*MYC* gene region) and 11q23 (*MLL* gene region) respectively, neither of which could have been deduced from routine cytogenetics. Both cases were accompanied by an unbalanced t(5;17). Amplification of 8q24 (case 4) was shown by FISH to be present in double minutes (Fig 3.3.2-B) and 11q23 (case 7) was shown to be incorporated in two marker chromosomes (Fig 3.3.2-C).

By comparing CGH with conventional karyotyping results, four cases (cases 4, 7, 9 and 12) revealed unbalanced rearrangements involving chromosomes 5 and 17 with different break points, all accompanied, to a varying extent, by deletion of 5 and 17. In case 4, CGH revealed loss of 5q14-qter and 17p12-pter whereas conventional karyotyping revealed loss of most of the material from 5q and 17p resulting from an unbalanced t(5;17). One of these cases (case 12) was thought to have a dicentric rearrangement retaining the centromeres of both chromosomes, and two cases (cases 7 and 9) showed an unbalanced translocation with interstitial deletion of chromosome 5.

FISH results

The composition of chromosomal aberrations, marker chromosomes and ring chromosomes could be identified using FISH with specific probes which were chosen because of discordance between CGH and conventional karyotyping as shown in Table 3.3.4.

Cases 7, 9, 12, 13, and 14 showed the composition of the marker chromosomes to be complex. Some of these markers were shown to be derived from segments of several different chromosomes (Fig 3.3.2-D, E, F). In case 13, chromosomal material involving the entire Y chromosome including the centromere, originally thought to be missing from the karyotype, was detected by FISH and CGH in the marker chromosome (Fig 3.3.2-G). In case 12, a derivative chromosome 7 was identified by FISH and CGH as a dicentric t(7p;21p) resulting in partial loss of 7p and 21p (Fig 3.3.2-H).

Table 3.3.3 Comparison between CGH and cytogenetic analysis in 19 AML and AML / MDS patients

Case	FAB-type	Karyotype	CGH results		
			gains	losses	Amp.
1	M3	46,XY,t(15;17)[13] 46,XY [1]	–[b]	–	–
2	M2	46,XY	–	–	–
3		46,XY	–	–	–
4	M4	46-47,XY,-5, ?der(9)del(9)(p13)inv(9)(p13q21.1) der(17)t(5;17)(p13;p11.2),+r,8-13dmin [CP10]	–	5q14–qter 9p11-pter 17p12-pter	8q24
5	M4	46,XY	–	–	–
6	M3	46,XY,t(15;17)(q22;q21)	–	–	–
7	M1	44-47,XY,add(4)(q13), der(5)t(5;17)(q1?2;q1?1), del(7)(q22),add(10)(p13), ?idic(11)(q23),add(15)(p11),-17, add(18)(p11),add(20)(q11.2),-21, +r x3,+mar[CP10]	–	5q15-q31 7q22-q36 11p 17p	11q, esp,11 q23- qter
8	M1	Fail	–	–	–
9	(RAEB)t	40-43,XY,der(3)t(3;15)(q21;q22),-4,-5, -9, del(9)(p2), -15, add(17)(p11.2),-18, -20,-21,-22,+2r,+mar1,+mar2,+mar3[CP9] 46,XY[1]	19 21 22	4 5q11.2-q31 9q12-q32 18 17p	–
10	M1	46,XY	–	–	–
11	M4	45,XY,t(3;3)(q21;q26),-7	–	7	–
12	M1	43,XY,del(3)(p13),del(5)(q11.2), del(7)(p13p15) or add(7)(p13), der(8;17)(q10;q10),-18,-21,-22, +der(?)t(3;?)(p21)[CP7] 42-43,XY, add(1)(q?11),del(3)(p13), der(5)t(5;17)(q11.2;q11.2), del(7)(p13p15) or add(7)(p13),add(8)(p12),-18,-21,-22, +der(?)t(1;?)(q?21;?)[CP4] 46,XY[2]	–	5q11.2-qter 7p12-pter 8p11.2-pter 17p13 18q	–
13	M4	40-45,X,-Y,-5,-6,add(7)(q22),-8(x2), -16,-20,+r,+mar1,+mar2,+mar3,+mar4, +mar5[CP11] 46, XY [3]	6p21.1 8p21.2- q21.2	5q23-qter 6p22-pter 7q21.2-qter 16q	–

14	RAEB	45-47,XY,add(1)(q4?),-9,-11,-13, add(15)(p11),add(19)(p13),+mar1, +mar2, +r1, +r2[CP13] 46,XY[4]	19p	–	–
15		46,XY	–	–	–
16	(RAEB)t	47,XY,+mar [CP3] 46,XY [17]	16p 22	–	–
17		Fail	1p32-pter 9q31-qter 11q12-13.3 16p 17, 19, 20	5q11.1-q22 13q21 Xq	–
18		46,XY	–	–	–
19		46,XY	–	–	–

[a] CGH, Comparative Genomic Hybridisation; AML, Acute Myeloid Leukemia; MDS, Myelodysplastic syndromes; FAB, French-American-British, RAEB, Refractory Anemia with Excess Blast.

[b] no gain, loss or amplification

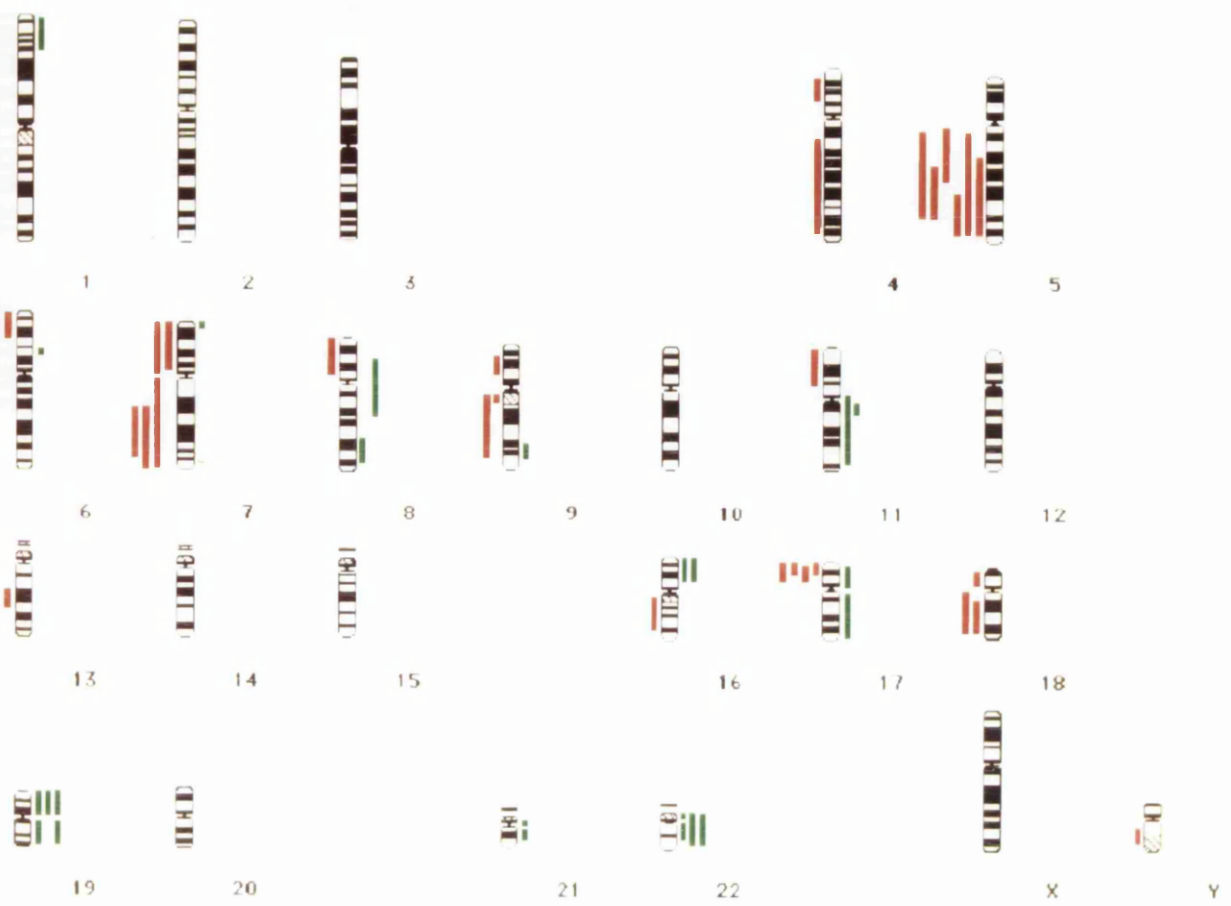


FIG 3.3.2-A Summary of CGH ratio profiles of 9 abnormal cases with AML/MDS; losses of DNA copy numbers are represented by vertical liines on the left side of each chromosome with red colour, whereas lines on the right side represent gains with green colour.

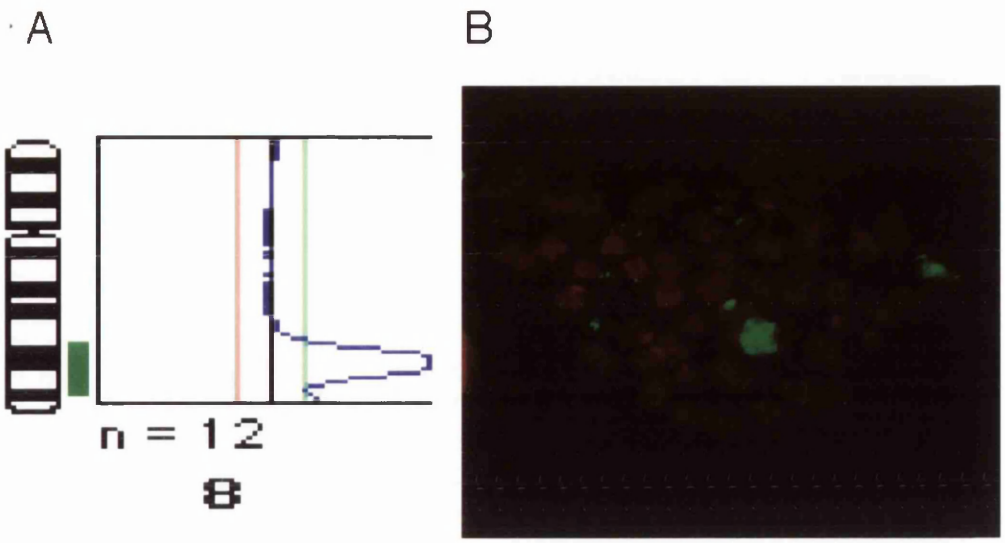


Fig 3.3.2-B CGH profile of chromosome 8 in case 4, which is represented amplification at 8q24 (A) and Mono-colour FISH hybridised on patient metaphase using WCP 8 (green). Result: Amplification of *C-MYC* incorporated in double minutes (B).

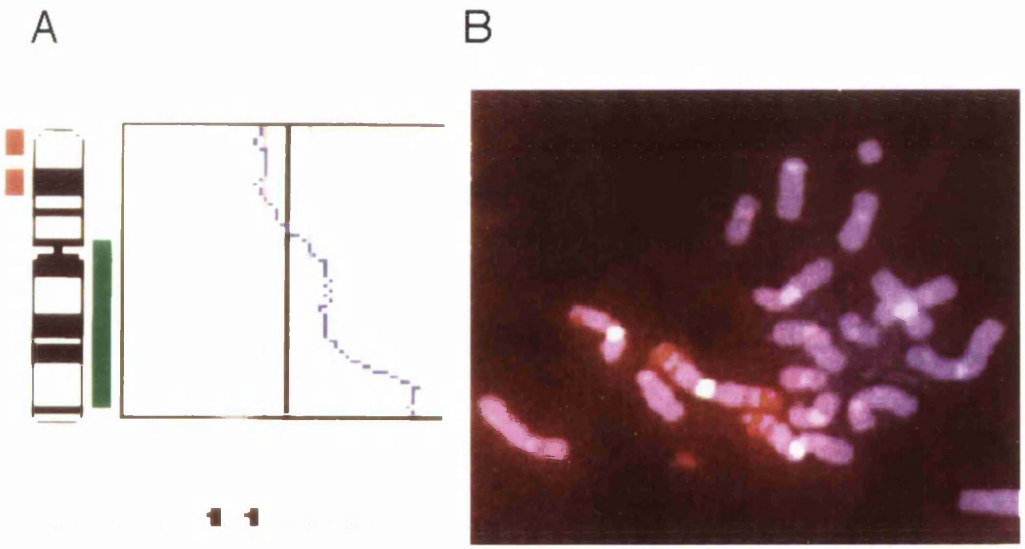


Fig 3.3.2-C CGH profile of chromosome 11 in case 7, which is shown amplification at 11q23 (A) and Mono-colour FISH hybridised on patient metaphase (case 7) using *MLL* gene specific probe (red). Result: 8 copies of the *MLL* gene on two der(11).

Table 3.3.4 The origin of unidentified regions within the karyotype identified by CGH/FISH

Case	Unidentified regions of the Karyotype	Origin of the unidentified regions	Probes used by FISH (WCP)[b]
4	+ r 8-13 dmin	5q 8q24	Ch 5 WCP Ch 8 WCP
7	mar 1(idic 11) mar 2(add 20) add (15) add (10)	11 4 11 4	Ch 11 WCP Ch 4 WCP Ch 11(B)/15(D) WCP Ch 4 WCP
9	mar 1 mar 2(add 17) mar 3 r 1 r 2	20 / 22 t(5;17) 3 9 / 21 15	Ch 20(D)/22(B) WCP Ch 5 WCP Ch 3 WCP Ch 9(B)/21(D) WCP Ch 15 WCP
12	add (7) der (?)	dic(7;21) 3 / 18 / 22	Ch 7(B)/21(D) WCP Ch 3(B)/18(D) +22(B)/18(D) WCP
13	mar 1 mar 2 mar 3 mar 4 mar 5	Y / 6 / 8 6 / 8 / 20 8 6 / 20 16	Ch 8(B)/6(D)+8(D)/Y(B) 8(B)/6(D)+6(D)/20(B) Ch 8 WCP Ch 6(D)/20(B) WCP Ch 16 WCP
14	mar 1 mar 2 r 1 r2 add (15) add (19)	11 / 19 11 / 19 11 / 19 11 11 / 19 9	Ch 11(B)/19(D) WCP Ch 11(B)/19(D) WCP Ch 11(B)/19(D) WCP Ch 11(B)/19(D) WCP Ch 11(B)/19(D) WCP Ch 9(B)/19(D) WCP

[a] CGH, Comparative Genomic Hybridisation; FISH, Fluorescence In Situ Hybridisation; dmin, double minutes

[b] Ch, chromosome; B, probe labelled with biotin; D, probe labelled with digoxigenin

WCP, FISH using whole chromosome painting

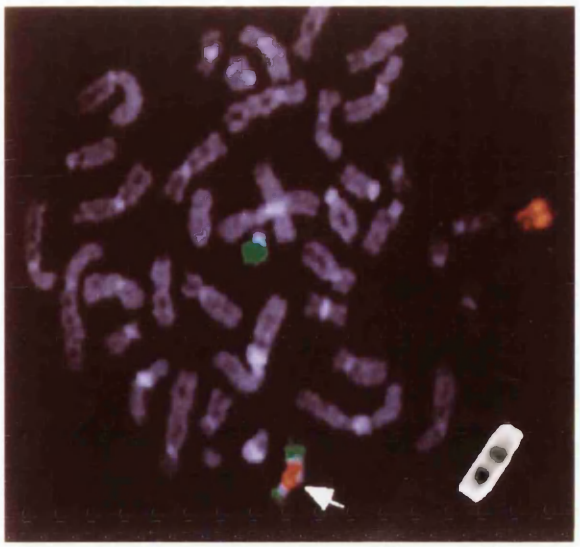


Fig 3.3.2-D Dual-colour FISH hybridised on patient metaphases (case 9). Probes used were WCP 20 (red) and WCP 22 (green). Results: The region of chromosome 20 and 22 in the make up of the marker chromosome (arrowed) is identified. It is possible that the marker chromosoem is an isoderivative chromosome 20. A G-banded image of the marker chromosome is also included.

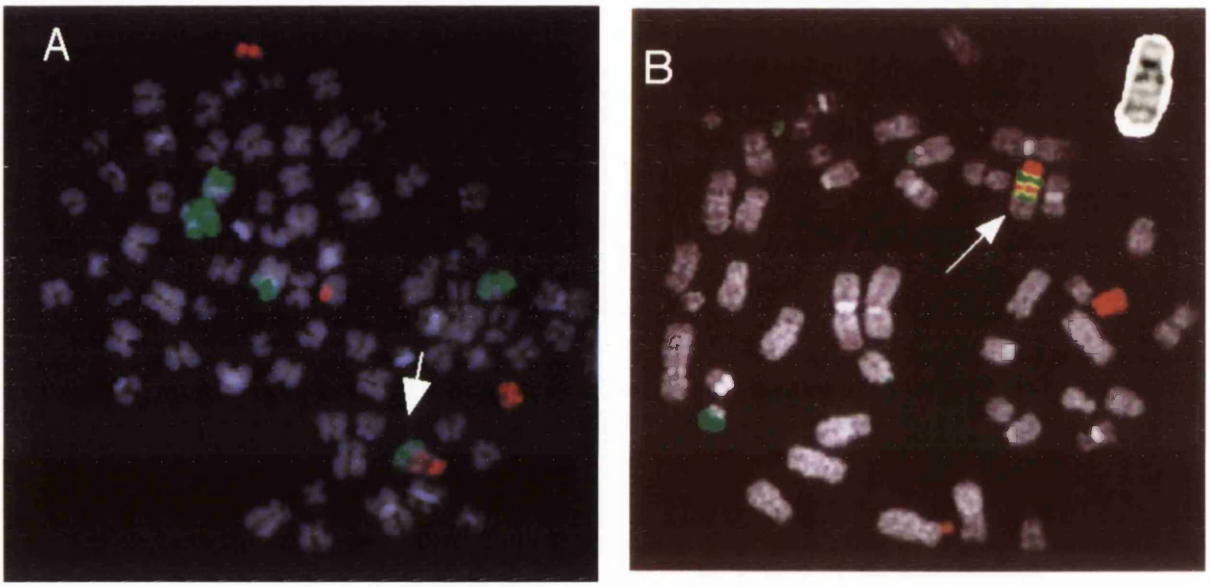


Fig 3.3.2-E (A)(B) Dual-colour FISH hybridised on patient metaphases containing the same cytogenic marker chromosome (case 12). (A) Probes used were WCP 3 (green) and WCP 18 (red). Result: The marker chromosome is composed of chromosome 3/18 material. (B) Probes used WCP 22 (green), WCP 18 (red). Results: The marker chromosome is composed of chromosome 22/18 material. A composite of both images shows the marker chromosome to be composed of regions from chromosome 3, 18, and 22. A G-banded image of marker chromosome is also included.

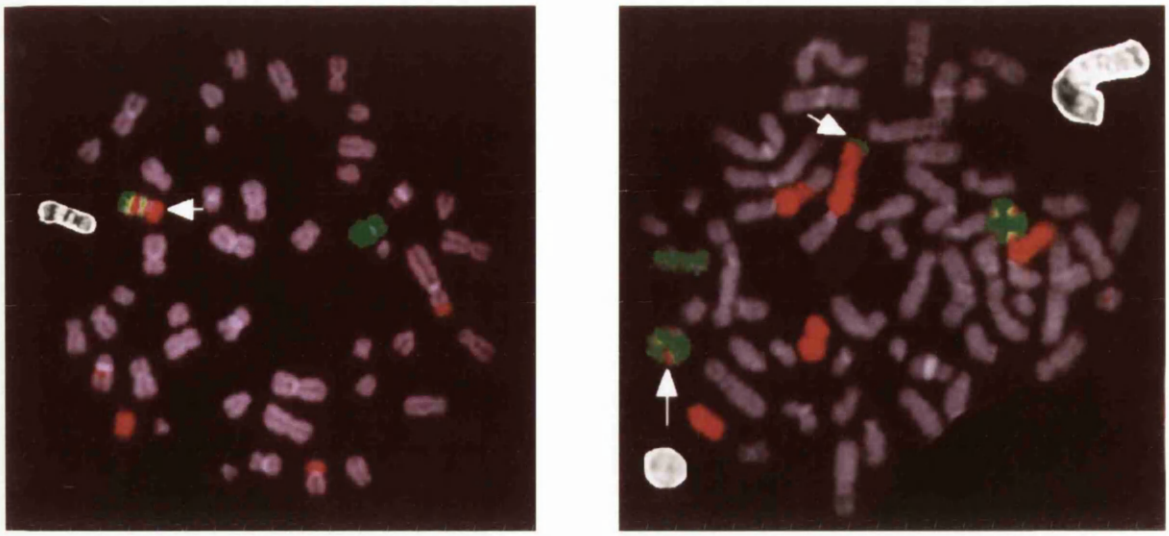


Fig 3.3.2-F (A)(B) Dual-colour FISH hybridized on patient metaphases (case 14). (A) Probes were used WCP 11 (green) and WCP 19 (red). Results: One marker chromosome shown as a G-banded image is composed mainly of chromosome 19 material with intervening sequences of chromosome 11. (B) Probes used were WCP 11 (green) and WCP 19 (red). Results: The additional material on chromosome 15 is identified as material from chromosome 11/19. The ring chromosome is composed mainly of chromosome 11 material interspersed with material from chromosome 19.

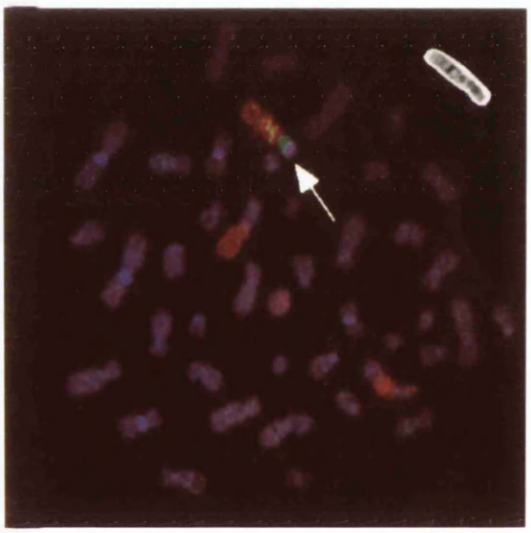


Fig 3.3.2-G Dual-colour FISH hybridized on patient metaphases (case 13) using WCP 8 (red) and WCP Y (green). Result: The marker chromosome (arowed) also shown as G-banded image, is composed of Chromosome 8 and Y materials.

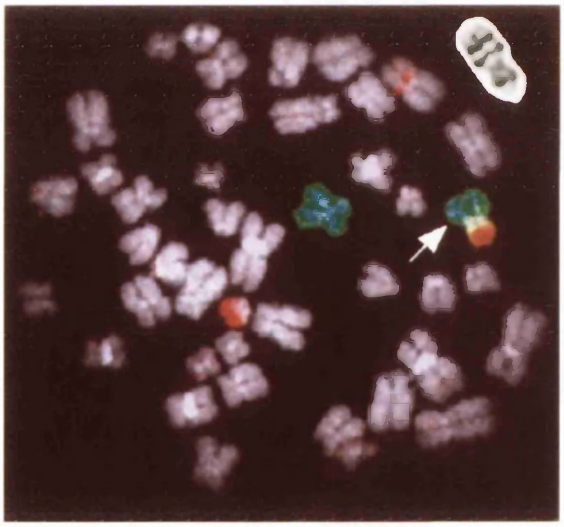


Fig 3.3.2-H Dual-colour FISH hybridized on patient metaphases (case 12) using WCP 7 (green) and WCP 21 (red). Results: The unidentified material on 7q (arrowed) also shown as a G-banded image is identified as chromosome 21 material. This chromosome was also identified as being dicentric.

3.3.3 CGH analysis on samples with various hematologic malignancies

Fourteen cases of various hematologic malignancies were analysed. A comparison of CGH and cytogenetic data is shown in Table 3.3.5. One of the documented limitations of CGH is that it cannot detect balanced abnormalities. Thus the Philadelphia chromosome was not detected by CGH in cases with CML. Case 9 & 11 showed chromosomal aberrations by G-banding analysis but CGH did not detect any abnormalities. Probably it was caused by mosaicism with normal cells (50%-70%).

In case 10 CGH revealed loss of 13q, compared with normal karyotyping result.

Table 3.3.5. Comparison between CGH and cytogenetic analysis in 14 various hematologic malignancies

No	Disease[a]	Karyotype	CGH
1	PRV	46,XY	Normal
2	PRV	46,XX	Normal
3	NHL	46,XY	Normal
4	NHL	46,XX	Normal
5	NHL	46,XX	Normal
6	Persistent leukocytosis	46,XX	Normal
7	MPD	46,XX	Normal
8	MPD	46,XX	Normal
9	CLL	46,XX,add(4)(q3?3), add(13)(q3?2),add(17)(p11.2)[3] 46,XX[9]	Cannot detect abnormality
10	CLL	46,XX	Loss of 13q
11	CLL	47,XY,+mar[2] 46,XY[28]	Cannot detect abnormality
12	Anemia	46,XX	Normal
13	CML	46,XX,t(9;22)	Cannot detect abnormality
14	CML	46,XY,t(9;22)	Cannot detect abnormality

[a] PRV, Polycythemia rubra vera; NHL, Non-Hodgkin's Lymphoma; MPD, Myeloproliferative disorders; CLL, Chronic Lymphocytic Leukemia; CML, Chronic Myeloid Leukemia.

3.4 CGH Experiments on fresh frozen solid tumour samples

The main aim of the project was to extend the study to the analysis of solid tumours first using fresh frozen samples and then moving on to use paraffin-embedded samples. It was particularly hoped to analyse early and sequential melanoma samples with the aim of highlighting chromosome regions involved in the onset of disease.

The first samples in this part of the study included 5 cases of well-differentiated liposarcoma (WDLS) and 17 cases of melanoma. Application of the CGH technique to the study of solid tumours has allowed a great improvement in identification of the genetic changes in various stages of cancer. CGH especially is the most convincing method to detect exact location of amplification on the chromosome, where a possible candidate oncogene may be located. The present study was planned to assess the usefulness of CGH in detecting net gains, losses, and amplifications of DNA copy number in solid tumour, which had not been detected by conventional karyotyping.

3.4.1 CGH analysis in cases with Well Differentiated Liposarcoma (WDLS)

Five samples from four individuals with WDLS investigated by the diagnostic laboratory were also studied by CGH using the standard method. All the samples yielded relatively high concentration, good quality DNA after extraction from the frozen samples except case 5 which was extracted from a cultured cell line. Chromosome analysis and CGH results are shown in Table 3.4.1.

One sample (case 5) gave a normal result by CGH. Because the DNA of this sample was extracted from cultured cell lines, it is possible that this reflects loss of the abnormal cell line during culture. All the remaining samples showed amplification of the chromosome region between 12q13 and 12q21 as a common chromosomal abnormality. But all three cases (cases 2 and 4; samples from same patient) showed slightly different locations and extent of amplifications. From the literature review, supernumerary ring and/or giant rod-shaped marker chromosomes were well-known frequent cytogenetic abnormalities in WDLS. These marker chromosomes are known to derive from material of chromosome 12, as well as less frequently from material from chromosome 1, 4, 8, 16 (Suijkerbuijk et al., 1994).

In case 1, the karyotyping result showed a hypotriploid/hyperdiploid clone including two giant derivative chromosomes. As already discussed, CGH cannot evaluate absolute copy numbers by standard normalization method. This case again showed that the computer software program readjusted internally to show 3 copies as the balanced state (Fig.3.4.1-A). But hypotriploidy/hyperdiploidy could be detected by applying single chromosome normalization. CGH results showed one copy of chromosome X, two copies of chromosome 2, 4, 9, 11(except 11p15.3; 4 copies), 14, 15, four copies of chromosomes 8, and 18, and three copies of the remaining chromosomes (except chromosome 5p15.3; 2 copies) in addition to amplification of 12q13-21. The discrepancies between CGH and conventional karyotyping result were two copies of chromosome 11(except 11p15.3; 4 copies), three copies of chromosomes 22 and 5 (except 5p15.3; 2 copies) and amplification of chromosome 12q13-21. After CGH, confirmatory FISH could not proceed because of unavailability of sufficient metaphase chromosomes. But it is likely that the gained and amplified materials could be present in the two giant derivative and marker chromosomes.

Cases 2 and 4 were from the same patient but taken at different times. Case 2 was the first sample approximately 4 months earlier than case 4. Conventional cytogenetics showed a ring chromosome to be present in 2 (case 2) or 3 copies (case 4). It was thought that these ring chromosomes were composed of material from chromosome 12. This interpretation was confirmed by FISH, which also showed that the chromosome 12 paint did not completely cover the rings. CGH analysis of these samples indicated that additional amplification material from chromosome 3 (3q12-13.3) was present (Fig 3.4.1-C). This amplification material could be present in the ring chromosomes, but unfortunately no material remained to confirm this by FISH.

The karyotype of case 3 when studied by cytogenetics contained 4 copies of a ring chromosome shown by FISH to be composed in part of material from chromosome 12. In addition to amplification of chromosome 12q21, two prominent peaks were detected at the 1q21-24 and 1q31 (amplification on chromosome 1q21-24, high level of gain on 1q31) by CGH and could be present in the ring chromosomes (Fig 3.4.1-B).

Table 3.4.1 Summary of cytogenetic and CGH results of 4 cases with WDLS

No	Karyotype	CGH
1	64,X,+1,+3,+add(5)(p14).ish der(5)(wcp12+), +6,+7,+8,+8,+10,+11,+12,+13,+16,+17,+18, +18,+19,+20,+21,+mar.ish der(?)(wcp12+)	Amplification of 12q13-21 1x X, 2x2/4/9/11(+11p15.3) /14/15, 4x 8/18, 3x others(-5p15.3)
2	48,XX,+2r 46,XX	Amplification of 12q13-21 Amplification of 3q12-13.3
3	48-50,XX,+4r[cp11] 46,XX	Amplification of 12q21 Amplification of 1q21-24, High level of gain of 1q31
4	49,XX,+3r 46,XX	Amplification of 12q13-21 Amplification of 3q12-13.3
5	47,XY,+r.ish r(wcp12) 46,XY	normal

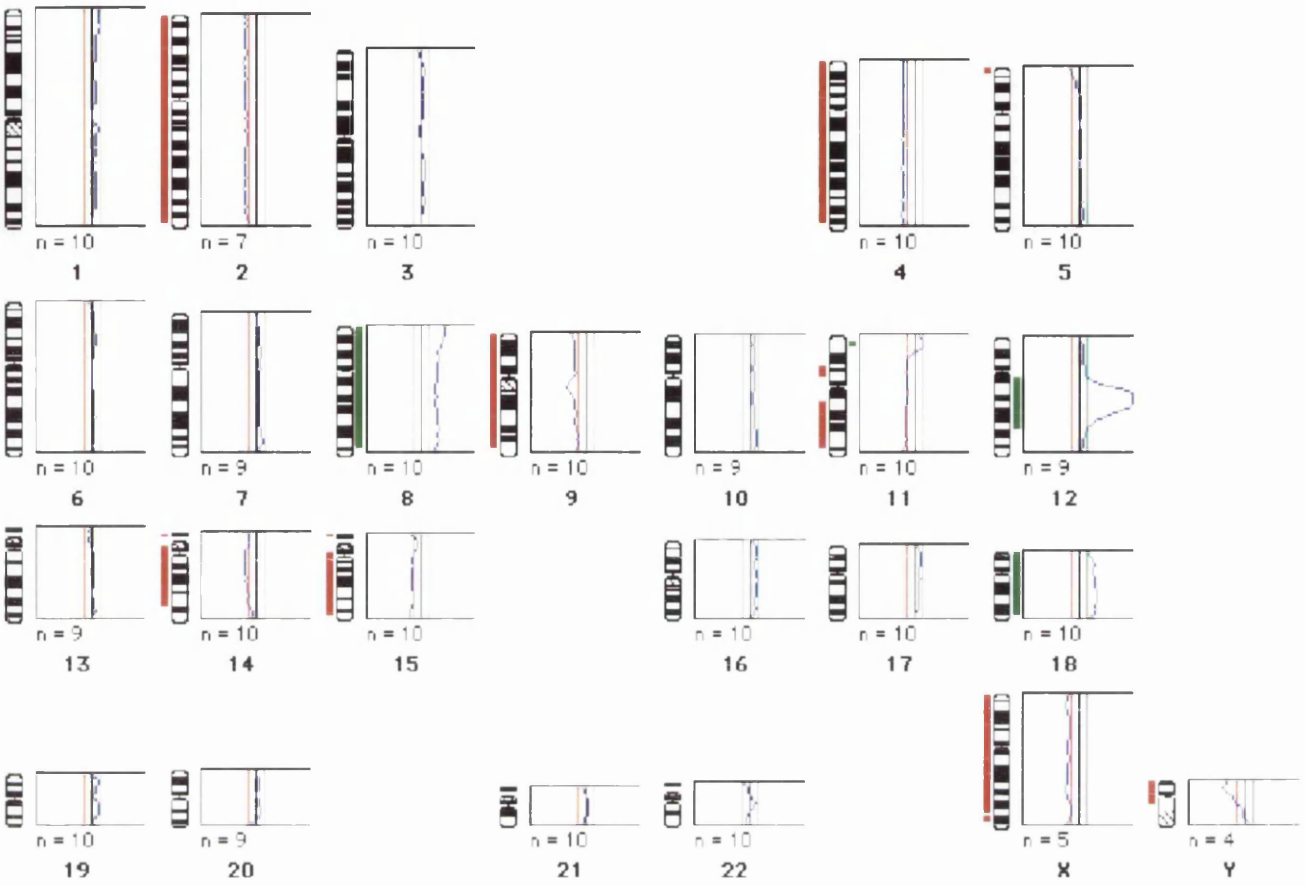


Fig 3.4.1-A CGH ratio profile from case 1 with well differentiated liposarcoma. This profile shows normal 2 copies of chromosome are represents as loss, 3 copies as balanced state, 4 copies as gain and in addition to amplification of 12q13-21.

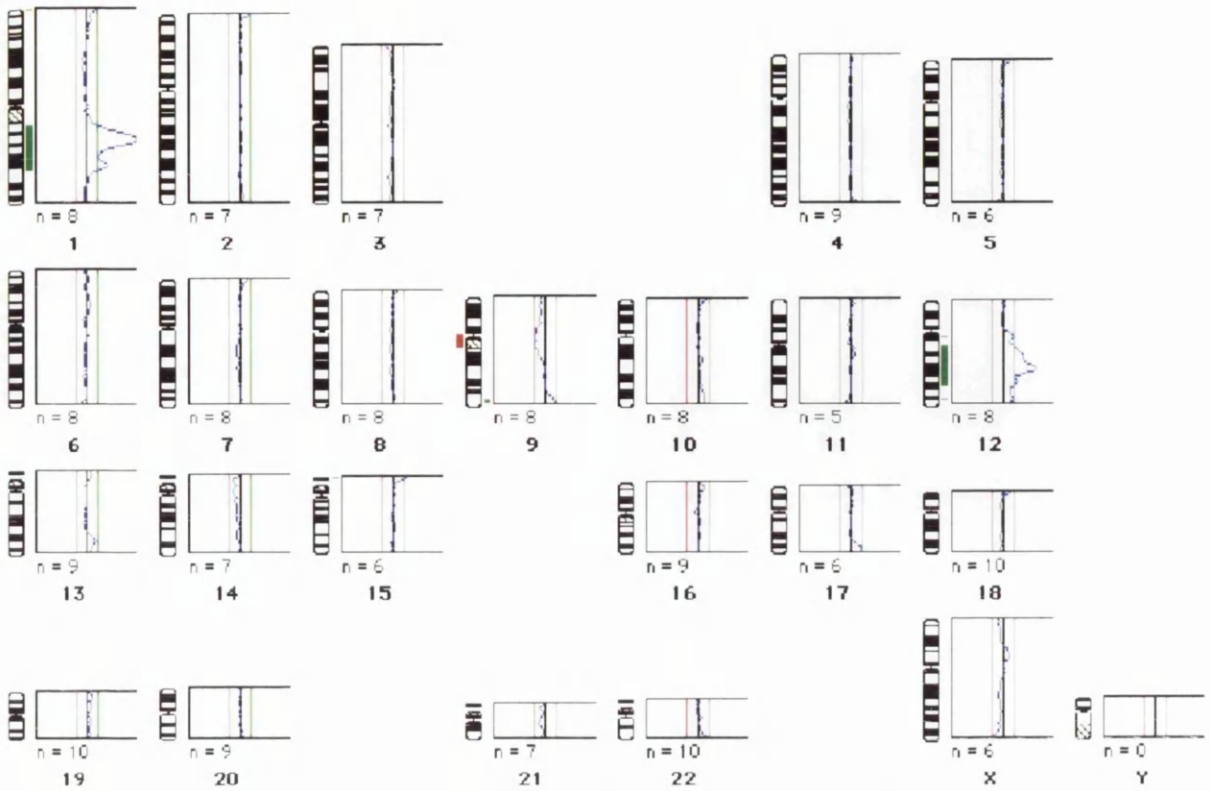


Fig 3.4.1-B CGH ratio profile from case 3. This profile shows amplification on chromosome 12q21, 1q21-24, and high level of gain on 1q31.

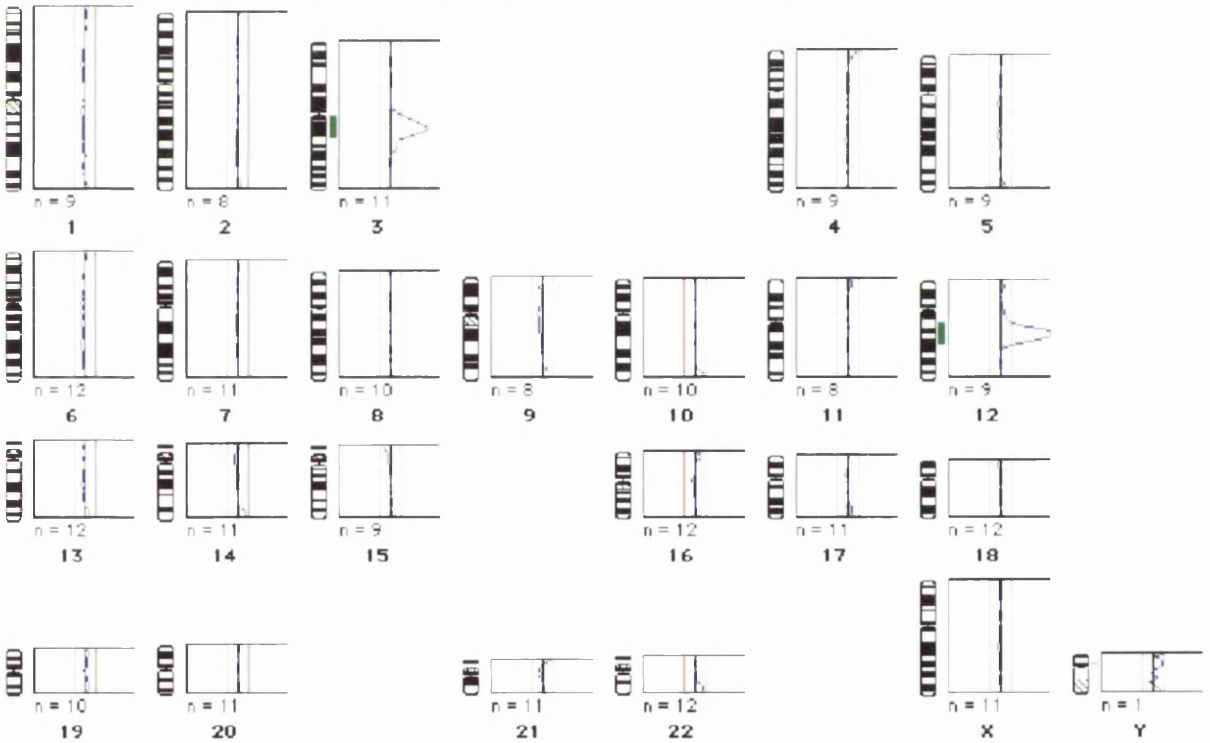


Fig 3.4.1-C CGH ratio profile from case 4. This profile shows amplification on chromosome 12q13-21 and 3q12-13.3.

3.4.2 CGH Analysis on Samples with Melanoma

In the first part of the melanoma study seventeen fresh frozen samples were studied. Because human cutaneous melanoma is one of the commonest cancers as well as the most malignant cancer, identification of genetic changes from early to metastatic stage is very critical. In samples of primary melanoma, it was very difficult to obtain pure malignant specimens because of contamination with normal surrounding tissue.

The aim of this study was to define chromosomes or chromosome regions which were frequently involved in karyotypic changes in early stages of the tumour. It was hoped that the application of CGH would help evaluate true net gains and losses and point to areas where a target gene involved in the progression of cancer might be located.

The seventeen samples studied were selected by the Dermatology department. The CGH experiments were performed without information about patient's status, histological subtype, or stage of tumour progression. No other cytogenetic analysis, including FISH, was possible on these samples.

A summary of the copy number changes in thirteen abnormal cases is shown as Fig 3.4.2-A. Losses of genetic material are represented by vertical lines on the left side of each chromosome, whereas lines on the right side represent gains. Remaining four cases showed normal CGH findings.

The most frequent finding was a loss of DNA segment on chromosome 10q with 10q11.2→q24 as the smallest under-represented segment (7 out of 17), a loss of 6q (7 out of 17), and loss of 9p (7 out of 17). Chromosome 10 was not always lost as the whole chromosome (loss of chromosome 10p; 5 out of 17). Losses of chromosome 6q were almost always accompanied with gains on chromosome 6p in our cases (6 out of 17, except case 14). In loss of chromosome 6q, the losses mostly involved the entire q arm with 6q16.1-q23.3 as minimal common lost area. Among cases with loss of 9p, six of the seven cases showed involvement of 9p21. One remaining case showed loss of 9p13.1-q22.1.

Another chromosomes showing frequent loss was 13q (5 out of 17). Cases 4, 10, and 14 showed loss of material from chromosome Y.

Gains most frequently involved chromosome 6p (7 out of 17), and this gain was of almost the entire p arm.

Other chromosomes showing frequent gains were p and/or q arms of chromosome 1 (7 out of 17), 8q (6 out of 17), 17q (6 out of 17), p and/or q

arms of 7 (5 out of 17), and 22 (3 out of 17). Gains of chromosome 1 were random over the whole length of the chromosome, but especially involved the region 1p33-36.1 (3 out of 17). In cases with gains on chromosome 8, 8q24 was the most frequently involved region (6 out of 17). Chromosome 7 showed inconsistent gains; the most commonly involved areas were 7q31.32-qter (5 out of 17) and 7q11.21 (4 out of 17). Gains on chromosome 17 mostly involved the 17q24-qter region.

In this study, high level of gains on chromosome 1q21, 11q12.3-q13.1 and amplifications on chromosome 1p11.2-p12, 3p24, 17q24-qter, and 22 were identified (threshold; > 1.5) (Fig 3.4.2-B). The degree of amplification of some regions appeared relatively low because the sizes of the amplicons were small and these samples were diluted with normal cells.

Two samples (case 6 and case 17) were from the same patient but taken at different times (time difference was 7 month 14 days). Case 6 showed gains of several small areas (11q13.2-13.4, 17q23-qter, +19), loss of 6q14.2-23.2, loss of 9p12-p22 and high level of gain of the region 3p24-25.2 including amplification on 3p24. After the gap in time, case 17 showed gains on chromosome 5q12-14, 5q21, 6p, +7 and losses on chromosome 2p, 6q, 9p22-q31.1, 13q22-34. These results are quite different from the original - all the regions showing gain in sample 6 had disappeared in sample 17. The features common to the two samples were loss of 9p21 and of 6q14.2-23.2.

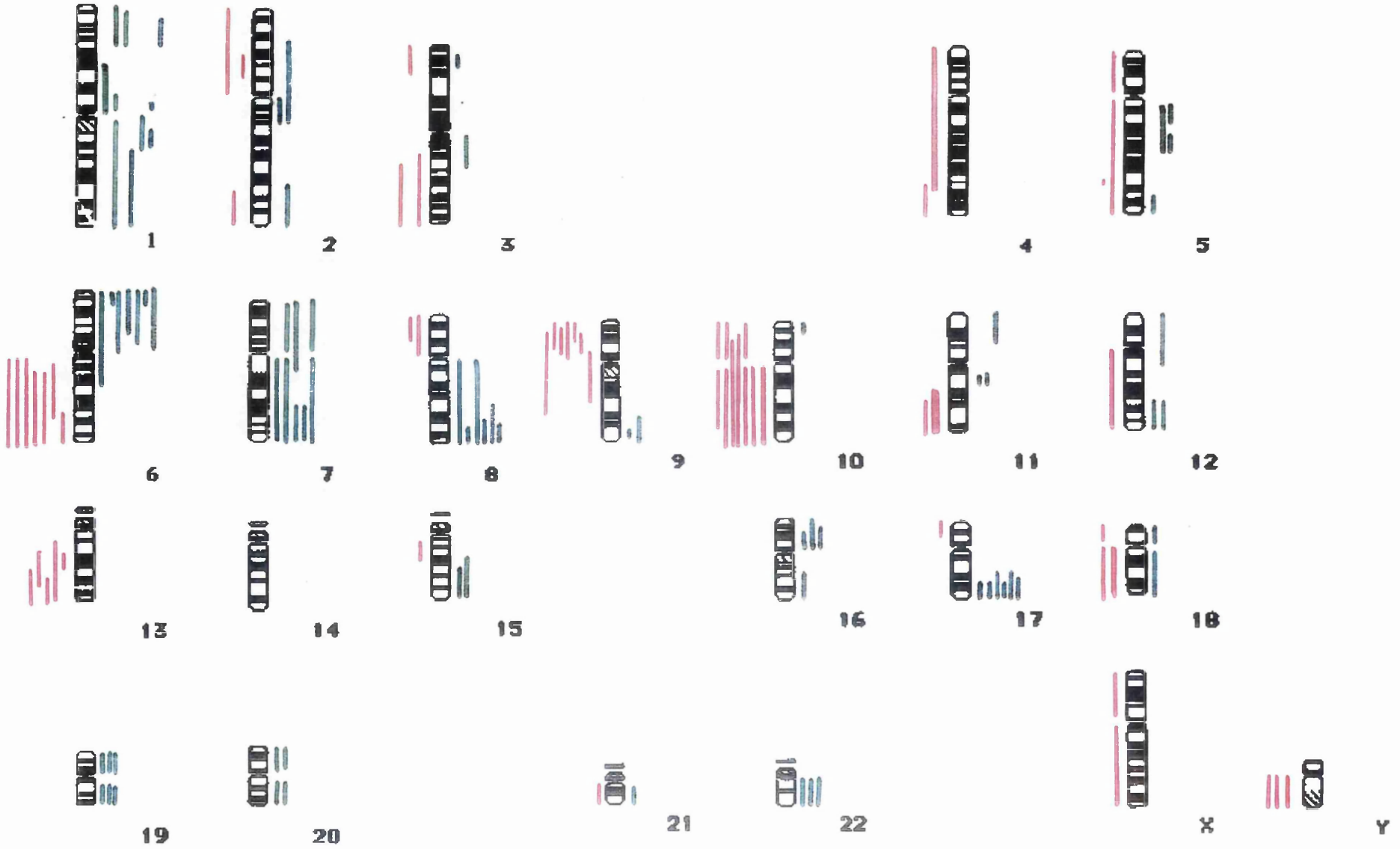


Fig 3.4.2-A Summary of the copy number changes in 13 abnormal melanoma cases

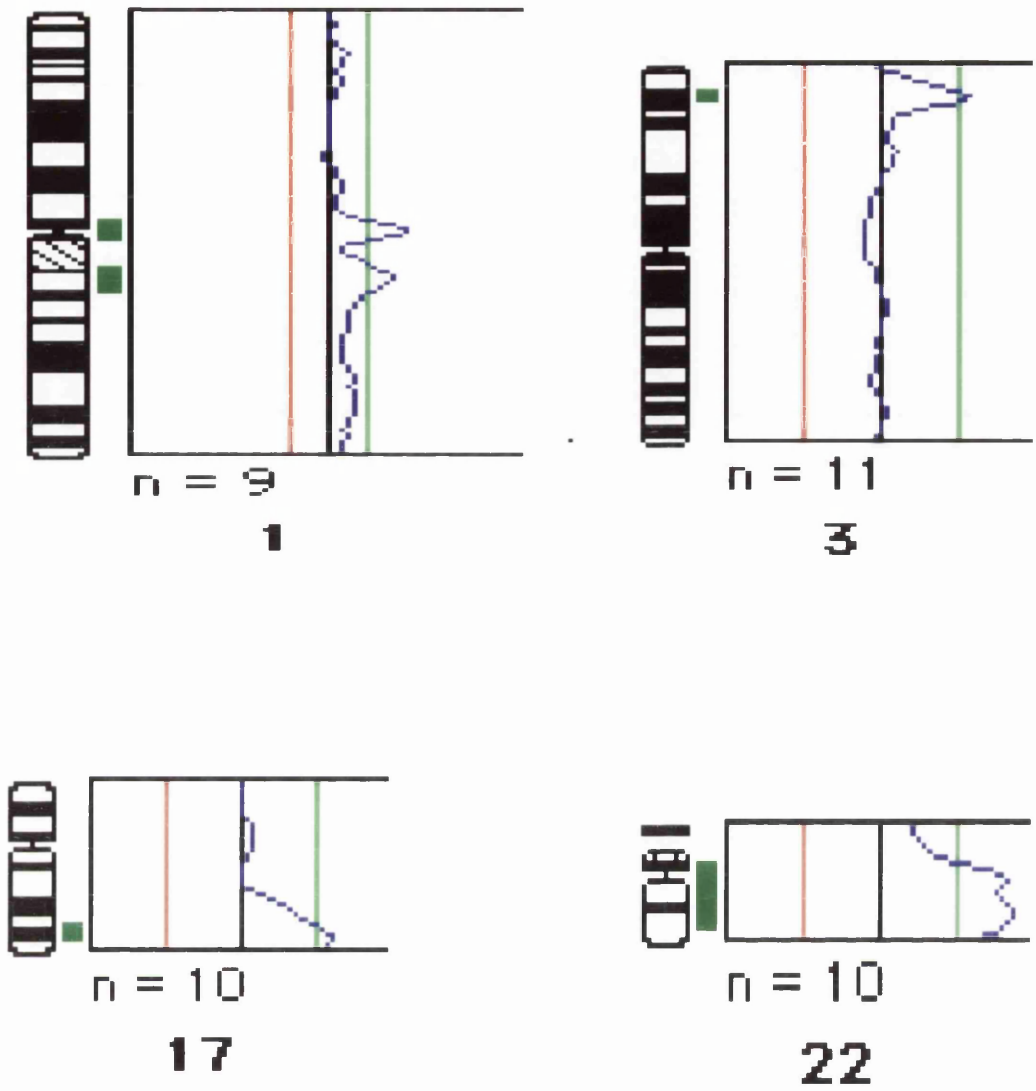


FIG 3.4.2-B Amplifications of four different regions on chromosomes 1p11.2-p12, 3p24, 17q24-qter, 22 in three melanoma cases

3.5 Optimisation of the CGH Technique in Very Small Amounts of DNA from Paraffin Embedded Tissue in Solid Tumours

CGH was developed to overcome the problem of cytogenetic analysis of solid tumours. Two of the problems of using sections are i) neoplastic tissue to be analysed may only be a small fraction of the cells in the specimen and ii) the amount of DNA obtained may be insufficient for CGH. The first problem can be overcome by separation of the malignant cells by microdissection, the second by whole genome amplification of the DNA obtained from microdissected malignant cells. CGH with amplified DNA from paraffin embedded tissue provides a powerful method to study the pathogenesis of cancer by identifying genetic progression from premalignant lesions to metastatic tumours (Speicher et al., 1993; Isola et al., 1994).

The samples used during optimisation were paraffin-embedded ovarian cancer tissue. The results of each modification were measured first by smear on the 1.2% agarose gel and secondly by ability to give good CGH after amplification. Even when the smear did not appear satisfactory amplification and CGH experiment were still attempted. The summary of the optimisation process in this experiment is present in Table 3.5.1.

The first step in the present project was to find the optimal method of production of high molecular weight DNA from formalin fixed, paraffin-embedded tissues.

3.5.1 Various kinds of DNA extraction methods from paraffin embedded tissue

Before starting CGH experiments with paraffin embedded samples, several modifications were investigated to find the optimal method for production of high molecular weight DNA for proceeding to whole genome amplification to obtain successful CGH.

Table 3.5.1 Summary of the optimization process in this experiment

No of DNA Isolation method	Souce of samples	Size of sample	Method of amplification		[c]Enzyme used	Result of CGH
			[a]PEP	[b]DOP		
1	Ovarian ca	1x7μm				
2	Ovarian ca	1x7μm	PEP-1		AmpliTaq pol	failed
				DOP-1	Topo + T7	failed
				DOP-2	T7	failed
3	Ovarian ca	1x7μm				
4	Ovarian ca	3x7μm	PEP-2		AmpliTaq pol	failed
				DOP-3	AmpliTaq LD	failed
				DOP-5	Topo + T7	good
5	Ovarian ca	3x7μm		DOP-4	AmpliTaq LD	inadequate
6	Ovarian ca	1x7μm				
7	Ovarian ca	1x7μm				
8	Ovarian ca	1x7μm				
9 –1	Ovarian ca	1x7μm	PEP-3		AmpliTaq pol	failed
9 –2	Ovarian ca		PEP-3		AmpliTaq pol	inadequate
			PEP-11		AmpliTaq LD	good
10 –1	Ovarian ca	1x7μm	PEP-4		AmpliTaq LD	good
			PEP-6		AmpliTaq LD	failed
10 -2	Ovarian ca		PEP-5		AmpliTaq pol	failed
			PEP-7		AmpliTaq pol	failed
11 -1	Ovarian ca	1x7μm	PEP-8		AmpliTaq LD	inadequate
11 –2	Ovarian ca		PEP-9		AmpliTaq LD	inadequate
11 -3	Ovarian ca		PEP-10		AmpliTaq LD	failed
12	Ovarian Cancer	1x7μm	PEP-12		AmpliTaq LD	good
				DOP-6	Thermoseque nase	good
13	Ovarian Cancer	1x7μm	PEP-13		AmpliTaq LD	good
				DOP-6	Thermoseque nase	good
14	melanoma	1x7μm	PEP-14		AmpliTaq LD	*
				DOP-7	AmpliTaq LD	*
15	melanoma	1x7μm	PEP-15		EHF PCR	*
				DOP-8	EHF PCR	*

a; PEP, primer-extension preamplification b; DOP-PCR, degenerate oligonucleotide-primed PCR

c; pol; polymerase, Topo; topoisomerase 1, T7; T7 sequenase, EHF; Expand High Fidelity PCR

* The results were depended on the samples.

The quality and quantity of extracted DNA depends on the status of the samples including the fixative used in the tissue processing, the duration of the fixation, the age of the paraffin block and the size of the DNA fragment to be amplified. In the work described here, all the tumour samples were provided from outside the department and there was no opportunity to vary the fixation etc., so that it was not possible to make any comparison of basic conditions.

The basic protocol was method 2 used by Isola et al(1994). In this experiment, the parameters investigated were amount of test material, pre-incubation before de-waxing or not, changing composition of lysis buffer, changing concentration or length of incubation in proteinase K, adding booster proteinase K or not, extraction with phenol/chloroform or not. The DNA extraction from paraffin-embedded tissue has some limitations as the formalin fixation of the tumour tissues results in tissue DNA forming tight complexes with proteins, and being fragmented. Nuclear proteins are also extensively cross linked with each other (Isola et al., 1994).

For each parameter, Table 3.5.2 shows modifications and Table 3.5.3 shows results of experiments of each method.

First of all, comparing the quantity of test material (Table 3.5.2; isolation methods 3 and 4), 3 x 7 μm thickness sample (method 4) showed better quality of DNA (from over 12000 bp to less than 500 bp) than using 1 x 7 μm sections.

Methods 5, 6, and 7 included a preincubation step (from 3 hrs to overnight) before dewaxing. DNA fragments produced by method 5 showed higher molecular weight DNA (1000 to over 12000 bp) with stronger intensity than that of method 4. Methods 6 and 7 showed that the samples were dried slightly after pre-incubation over 6 hours.

In experiments 9 and 10, three parameters were changed- lysis buffer, amount and incubation time in proteinase K, and phenol/chloroform extraction. Lysis buffer was changed to 1x PCR buffer 11 (10mM Tris HCl pH 8.3, 50mM KCl) because SDS has an inhibitory effect on PCR. The amount of proteinase K was increased (0.3 mg/ml→ 1-2 mg/ml) and incubation time was decreased (55°C for 3 days→ 65°C for 90 min) from method 9. In methods 9 and 10, there was no significant difference in results between different amount of proteinase K (2mg/ml and 1mg/ml). Finally in experiments 9-2 and 10-1 phenol-chloroform extraction step was omitted. In experiments 9-1 and 10-2 (with phenol/chloroform extraction), a less strong smear on the agarose gel was obtained rather than in 9-2 and 10-1 (without phenol/chloroform extraction).

Afterwards, the phenol-chloroform extraction procedure was omitted during isolation of DNA as DNAs in these samples were of relatively small amount. It was better to use a single step extraction method than a two step method and remnant phenol, used for the removal of protein such as proteinase and nuclease, also had an inhibitory effect on PCR. In experiments 9-2 and 10-1, the results showed great improvement in the size of extracted DNA (most fragments >1000 bp) and the success rate in CGH experiments.

In method 11, different incubation times (90 min, 3 hours, 24 hours) and temperature (65°C and 55°C) of proteinase K were applied on the same sample and results showed no significant difference among the three different modifications.

With completion of method 12, the DNA extraction method was finalised with the exception of further alterations in the lysis buffer.

In method 13, lysis buffer with high concentration of Tris HCl and EDTA was used. The result did not give a strong smear when the DNA was run on agarose gel probably because EDTA with high concentration ($\geq$ 0.5 mM) also has an inhibitory effect on PCR.

Finally, from method 14 DNA extraction procedure moved on to investigate the microdissected samples from paraffin-embedded melanoma tissue. Depending on the enzyme used to be (Ampli-Taq DNA Polymerase LD or Expand High Fidelity DNA Polymerase), different lysis buffers were used in the DNA extraction procedure.

In conclusion this series of experiments determined the optimal conditions for DNA extraction from paraffin embedded sections.

Table 3.5.2 Summary of different DNA isolation methods

No.	Amount of test material	Preinc. Before dewax.	Lysis buffer	Proteinase K		Protein aseK booster	Phenol/ chlor. E xt.
				Amount	Inc. time		
1	1x7μm	No	500ul nuclei lysis buffer 0.5% SDS	0.4mg/ml	10 hrs (55°C)	No	Yes (x2)
2	1x7μm	No	100mmol/L Nacl 10mmol/L Tris Hcl 25mmol/LEDTA 0.5% SDS	0.3mg/ml	3 day (55°C)	0.5mg/ml 24hr,48hr	Yes
3	1x7μm	No	Same as No. 2	0.3mg/ml	4 days (55°C)	0.5mg/ml 24hr,48hr	Yes
4	3x7μm	No	Same as No. 2	0.3mg/ml	4 days (55°C)	0.5mg/ml 24hr,48hr	Yes
5	3x7μm	3hrs at 65°C	Same as No. 2	0.3mg/ml	4 days (55°C)	0.5mg/ml 24hr,48hr	Yes
6	1x7μm	6hrs at 65°C	Same as No. 2	0.3mg/ml	3 days (55°C)	0.5mg/ml 24hr,48hr	Yes
7	1x7μm	Overnight at 65°C	Same as No. 2	0.3mg/ml	3 days (55°C)	0.5mg/ml 24hr,48hr	Yes
8	1x7μm (M)	No	Same as No. 2	1mg/ml	1 day (55°C)	No	No
9	1x7μm	No	1xPCR buffer11	1mg/ml	90min (65°C)	No	9-1;Yes 9-2;No
10	1x7μm	No	1xPCR buffer11	2mg/ml	90min (65°C)	No	10-1;No 10-2; Yes
11	1x7μm	No	1xPCR buffer11	2mg/ml	11-1;90min(65°C) 11-2;3hrs(65°C) 11-3;24hrs(55°C)		No
12	1x7μm	No	1xPCR buffer11	1mg/ml	24hrs (55°C)	No	No
13	1x7μm	No	50mM TrisHcl 1mM EDTA 0.5% Tween 20	1mg/ml	24hrs (55°C)	No	No
14	1x3μm (M)	No	1xPCR buffer11	1mg/ml	2days (55°C)	No	No
15	1x3μm (M)	No	EHF buffer 0.5% Tween 20	4mg/ml	24hrs (48°C)	No	No

M; microdissection

Table 3.5.3 Results of different DNA isolation methods

No of DNA isolation method	Size of DNA fragments	Result of CGH	Method of amplification	Order of Intensity (smear)
1	Failure to get			
2	< 500 bp	failed failed	DOP-PCR & PEP	5
3	< 500 bp			5
4	200 to over 12000 bp	successful failed	DOP-PCR & PEP	4
5	1000 to over 12000 bp	inadequate	DOP-PCR	2
6	< 500 bp			
7	< 1000 bp			
8	Failure to get			
9 –1 9 –2	500-12000 bp 1000 to over 12000 bp	failed successful	PEP PEP	3 1
10 -1 10 -2	500- 12000 bp 500 to over 12000 bp	successful failed	PEP PEP	3 4
11 -1 11 –2 11 -3	1000 to over 12000 bp 1000 to over 12000 bp 1600-12000bp	inadequate inadequate failed	PEP PEP PEP	2 2 2
12	500-12000 bp	successful successful	DOP-PCR & PEP	1
13	500 to over 12000 bp	successful successful	DOP-PCR & PEP	4
14	*	* failed	DOP-PCR & PEP	*
15	*	* failed	DOP-PCR & PEP	*

order of relative intensity; very strong- moderate strong- strong- faint- very faint (1-2-3-4-5)

* The size of DNA fragments and intensity of smear and results of CGH vary in the original melanoma samples.

3.5.2 Universal amplification of DNA

Whilst the optimal method of DNA extraction was being determined, the next steps to whole genome amplification and CGH experiment were also evaluated. These methods were developed to allow genetic analysis to be undertaken on very small samples of DNA. Several trials have been described where CGH had been attempted after universal amplification using DOP-PCR but the technique required to be optimized, as did the alternative PEP.

To optimize the universal amplification method the following factors were investigated: cell lysis method (DNA extraction method), amplification cycles and enzymes.

Degenerate Oligonucleotide Primed PCR (DOP-PCR)

DOP-PCR is a type of PCR which uses primers which include partially degenerate oligonucleotides. The important points of DOP-PCR are the degenerate nature of primer and a low initial annealing temperature because these conditions induce priming from multiple evenly dispersed sites within a given genome.

The aim of this part of the study was to assess whether amplified DOP-PCR products could produce successful CGH results without significant bias and to evaluate the effect on DOP-PCR product of changing various parameters, which were the enzyme used, number of initial low annealing temperature cycles, and different kind and size of template DNA.

Table 3.5.4 shows some modifications during optimization of DOP-PCR and the results of different DOP-PCR methods are presented in Table 3.5.5. The first part of this experiment used samples of paraffin-embedded tissue from ovarian cancer (method number 1-6).

Table 3.5.4 Summary of DOP-PCR modification protocol

No	Amount of template (No. of Table 3.5.2)	topoisomerase	1st low annealing temp. cycles		1st high annealing temp. cycles		Conc. of DNA
			Enz.	Cycles	Enz.	cycles	
1	3μl 1x7μm (No.2)	+	T7sequenase	94°C 1min 30°C 1min30s 37° 2min x7	Ampli Taq LD.	94°C 1min 56°C 1min 72°C 3minx35	320ng /μl
2	3μl 1x7μm (No.2)	–	T7sequenase	Same as No.1	Same as No1	Same as No.1	220ng /μl
3	3μl 3x7μm (No.4)	–	Ampli Taq LD	94°C 1min 30°C 1min30s 37°C 2min x7	Same as No1	Same as No.1	460ng /μl
4	3μl 3x7μm (No.5)	–	Ampli Taq LD	Same as No.3	Same as No1	Same as No.1	320ng /μl
5	3μl 3x7μm (No.4)	+	T7sequenase	94°C 1min 30°C 1min30s 37°C 2min x7	Same as No1	Same as No1	50ng/ μl
6	3μl 1x7μm (No.12,13)	–	Thermo sequenase	94°C 1min 25°C 1min 74°C 2min x4	Ampli Taq LD	94°C 1min 56°C 1min 72°C 2minx30	450ng /μl
7	5μl 1x3μm	–	Ampli Taq LD	93°C 1min 30°C 1min 72°C 3min x7 or x8	Ampli Taq LD	93°C 1min 60°C 1min 72°C 3minx28	
8	5μl or 10μl 1x3μm or 1x7μm, microdissected	-	EHF DNA polymerase	93°C 1min 30°C 1min 72°C 3min x8	EHF DNA polymerase	93°C 1min 60°C 1min 72°C 3minx28	

Table 3.5.5 Results of different DOP-PCR methods

No of DOP-PCR method	Size after first DOP-PCR	Size after 2nd DOP-PCR	*Order of intensity on smear (1st)	CGH results
1 (Fig 3.5.2-A)	from over 12000 to below 500 bp	< 500 bp	2	fail
2 (Fig 3.5.2-A)	from 3000 to below 500 bp	< 500 bp	3	fail
3 (Fig 3.5.2-C)	from 2000 to below 1000 bp	mainly 200 to 500 bp	3	fail
4 (Fig 3.5.2-C)	from 12000 to below 1000 bp	200 to 1000 bp	2	inadequate
5 (Fig 3.5.2-E)	from over12000 to below 500 bp	300 to1000 bp	1	good
6 (Fig 3.5.2-G)	from 12000 bp to below 500 bp	300 to 500 bp	1	good
7 (next chapter)				

* the order of relative intensity from strong to very faint (1→ 2→3)

Although DOP-PCR methods 1 and 2 were performed with the same amount of DNA from the same sample, method 1 employed topoisomerase and T7 sequenase in 7 cycles with lower annealing temperature. Method 2 used T7 sequenase without topoisomerase 1. One function of topoisomerase 1 is to unwind the supercoiled DNA structure. In this study, topoisomerase 1 was not expected to produce a marked effect. Because the DNA in an interphase cell has a less condensed structure the efficiency of initiation complex formation is better than that in metaphase chromosomes due to increased access of primer and polymerase to the genomic template DNA. T7 sequenase functions well at lower annealing temperature to stabilize the random priming complexes and can synthesize long stretches of DNA by displacing other primers that have already annealed to the DNA. However, the method using T7 sequenase involved repeated pipetting because of the heat-labile nature of this enzyme and hence had an increased chance of contamination. Comparing both results, method 1 gave longer fragments of DNA and a slightly stronger smear on standard agarose gel than those of method 2, but both of them showed distinct bands around 400 bp region (Fig 3.5.2-A). Both methods on CGH showed less intensity of green (test DNA) in comparison with red (control DNA). Intensity was further reduced over regions corresponding to dark G-bands. Over-representation of some telomeric regions, and distal part of chromosome 1p, 16p, 19, and 22 was observed. Neither method could obtain any analysable CGH images (Fig 3.5.2-B). It was thought that DNA amplified by these methods was too fragmented and at too low a concentration to produce proper CGH results.

Methods 3 and 4 used increased starting material (3 x 7 μm) for Whole Genome Amplification (WGA) in a similar way to method 2 in DOP-PCR protocol (no topoisomerase 1, with AmpliTaq LD instead of T7 sequenase). The only difference between method 3 and 4 was the DNA isolation method (DNA extraction method 3; without preincubation, method 4; with preincubation). In comparison of method 3 with 4, both showed a faint smear but method 4 showed a slightly longer, stronger smear than method 3 after first DOP-PCR (3.5.2-C). Both products were used for CGH after labelling by nick translation. Only method 4 gave CGH images with matched sex chromosomes (test DNA; female, reference DNA; male hybridised on female metaphase) which showed less prominent incorrectly over-represented regions and banded pattern (Fig 3.5.2-D).

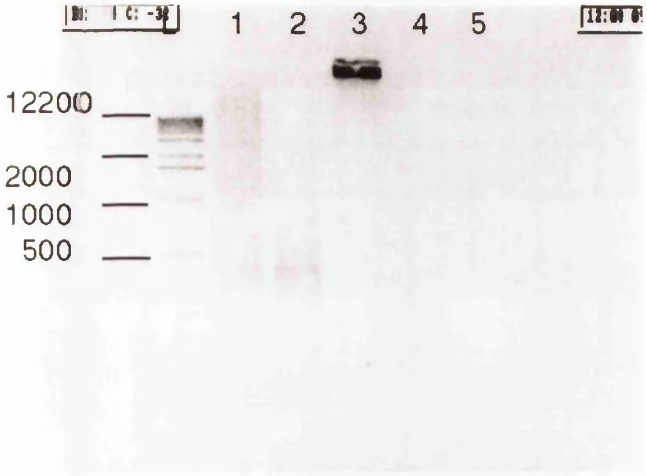


FIG 3.5.2-A

Gel electrophoresis of the first DOP-PCR products amplified by method 1 & 2

L; Ladder
1; DOP-PCR method -1
2; DOP-PCR method -2
5; negative control

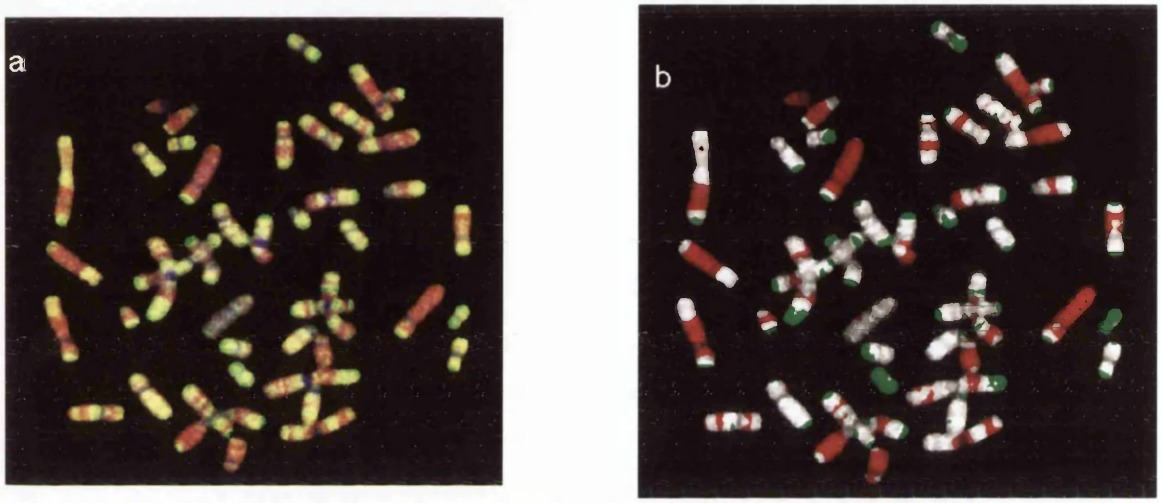


FIG 3.5.2-B a) CGH image following colour(fluorescence) equalisation and b)colour ratio image using product amplified by DOP -PCR method 1

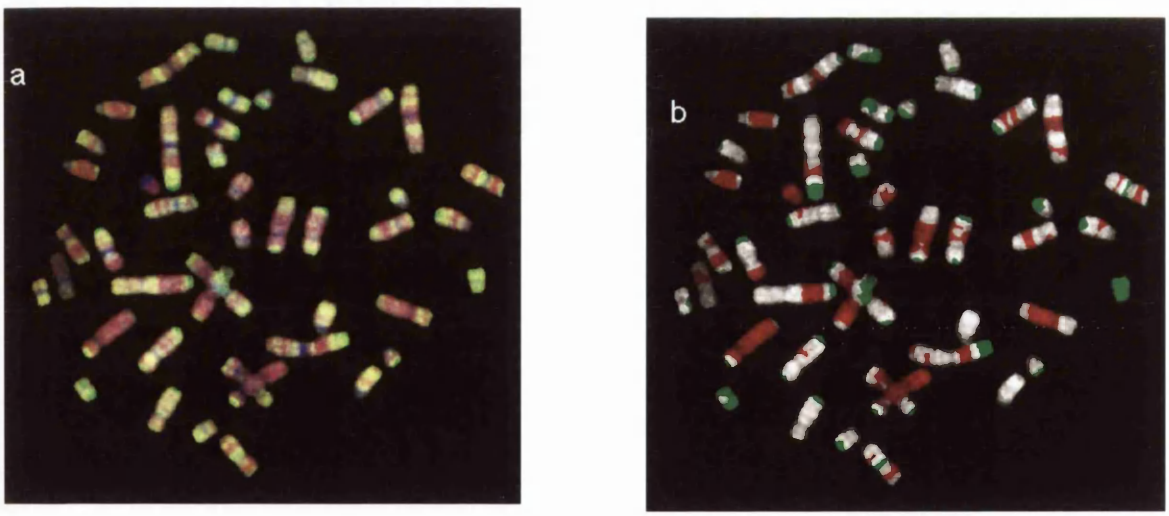


FIG 3.5.2-B a) CGH image following colour (fluorescence) equalisation and b) colour ratio image using product amplified by DOP -PCR method 2

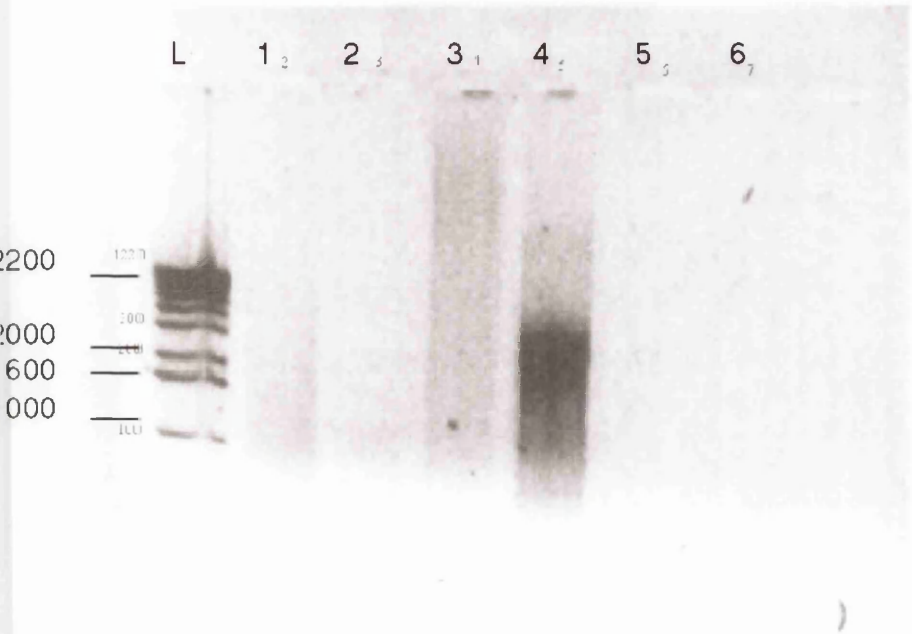


FIG 3.5.2- C

Gel electrophoresis of the first DOP-PCR products amplified by method 3 & 4

L; 1 kb ladder
1; DOP-PCR method -4
2; DOP-PCR method -3
6; negative control

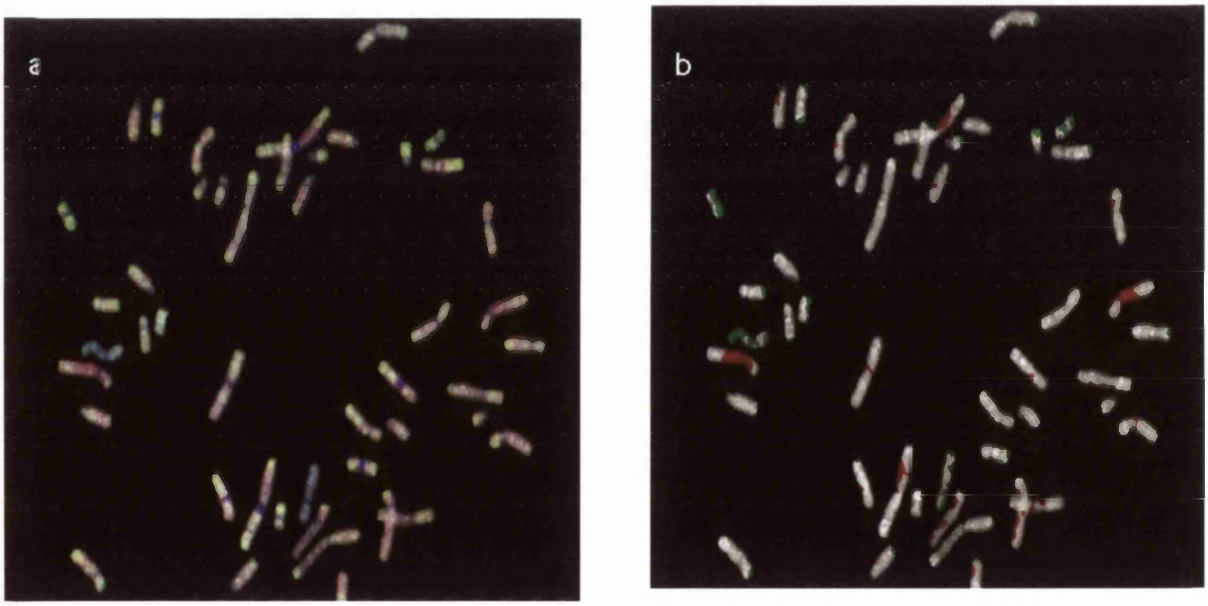


FIG 3.5.2 - D a) CGH image following colour (fluorescence) equalisation and b) colour ratio image using product amplified by DOP-PCR method 4

To avoid further fragmentation during nick translation, the labelling method was changed from method 5 onwards. DOP-PCR labelling was applied during the second or the third rounds of PCR.

Method 5 was performed on the same DNA as was used in DOP-PCR method 3 and the method used in the first part of the experiment was that employed in DOP-PCR method 1, but labelling was carried out during the second PCR rather than by nick translation. The DNA size of the product after the first DOP-PCR was from very small fragment to over 12000 bp (especially over 3000 bp) (Fig 3.5.2-E). After the second PCR with labelling, DNA size was relatively ideal for CGH (approximately 300 to 1000 bp). The CGH result showed more homogeneous hybridisation than the previous ones and some abnormal regions with matched sex chromosomes (test DNA; female, reference DNA; male hybridized on male metaphase) (Fig 3.5.2-F).

Method 6 applied another protocol with minor modifications (Kuukasjärvi et al., 1997) to a DNA sample of 1x7μm thickness of paraffin-embedded ovarian cancer tissue using thermosequenase for decreasing the chance of contamination. The DNA size of amplified products after the first DOP-PCR was from 300 bp to 12000 bp with a distinct band at 400-500 bp region (Fig 3.5.2-G). Relatively smaller sized DNA (300-500 bp) with a strong intensity on agarose gel was seen after the second PCR with labelling. CGH showed analysable good images for the first time, but this result still had a problem, in that the CGH image appeared still slightly granular with over-represention of some telomeric regions including chromosome 19 and 22 (Fig 3.5.2-H).

Using method 6, DOP-PCR was applied to various samples including some melanoma samples, serially diluted DNA from normal female blood, abnormal bone marrow samples and thick sectioned paraffin embedded ovarian cancer tissue. Successful results were not always obtained because there are many variables during the stages from isolation of DNA to the end of CGH with many critical steps at each stage of the process.

To obtain more consistent CGH results, some modifications were tried including increasing the number of the first low annealing temperature cycles, extension time, and decreasing the number of high annealing temperature cycles to increase efficacy of DOP-PCR and to reduce error during PCR. However it was felt that by this time the DOP-PCR was fairly well established and that the results were reflecting differences in the sample itself not in the CGH procedure.

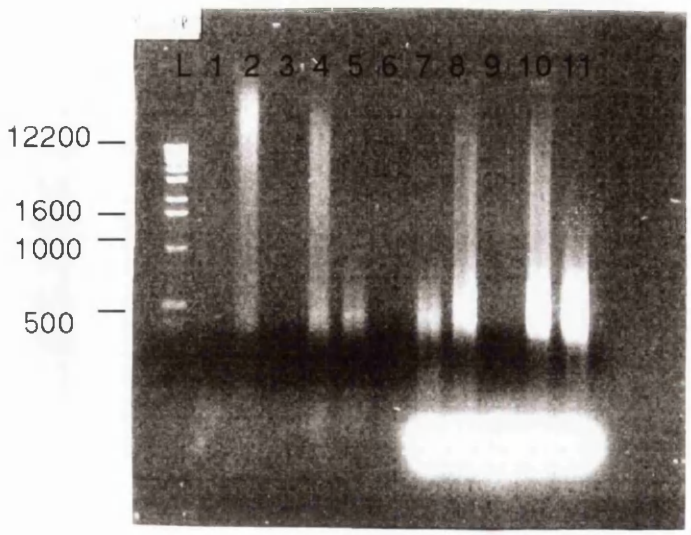


FIG 3.5.2- E

Gel electrophoresis of the first DOP-PCR product amplified by method 5

L; 1 kb ladder
2; DOP-PCR mehtod -5
6; negative control

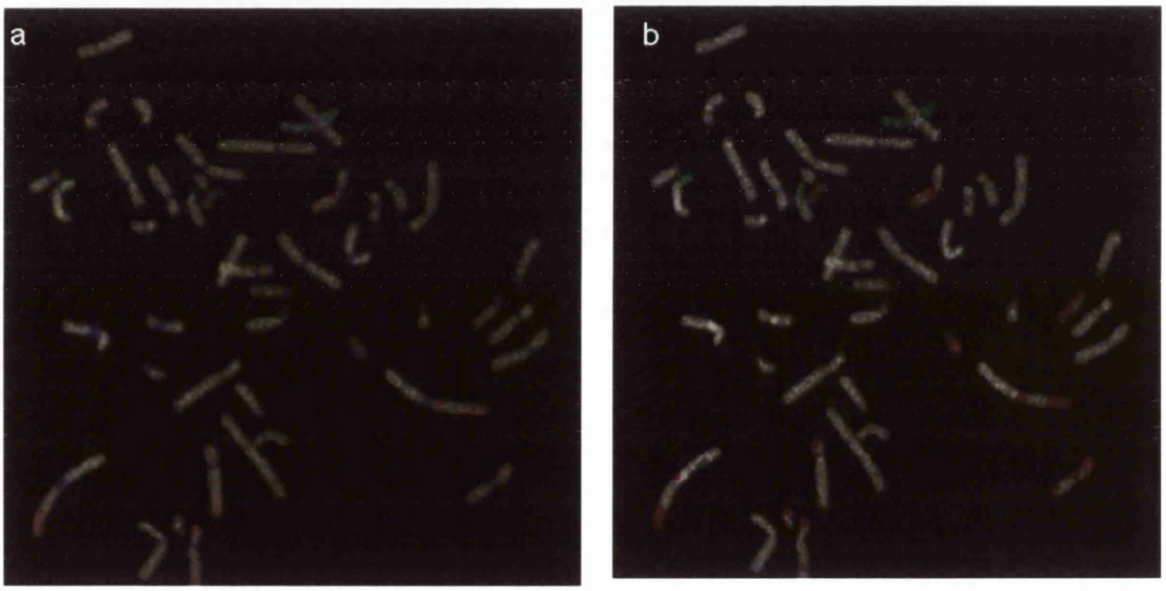


FIG 3.5.2-F CGH image following colour equalisation (a) and colour ratio image (b) using product amplified by DOP-PCR method 5

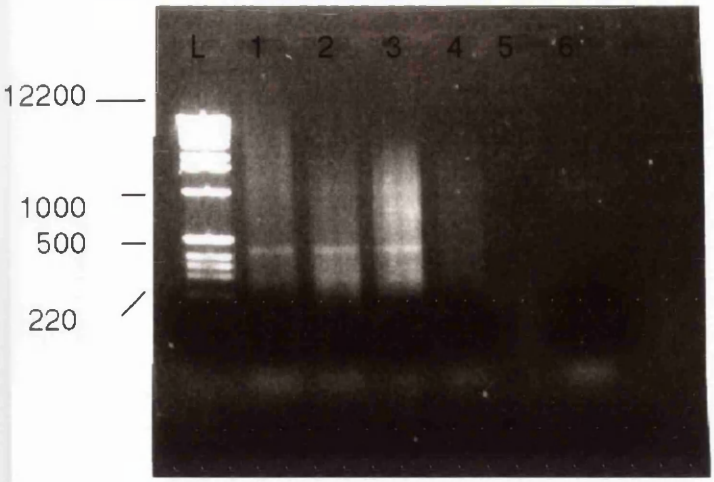


FIG 3.5.2-G

Gel electrophoresis of the first DOP-PCR product amplified by method 6

L; 1 kb ladder
1; DOP-PCR method - 6
6; negative control

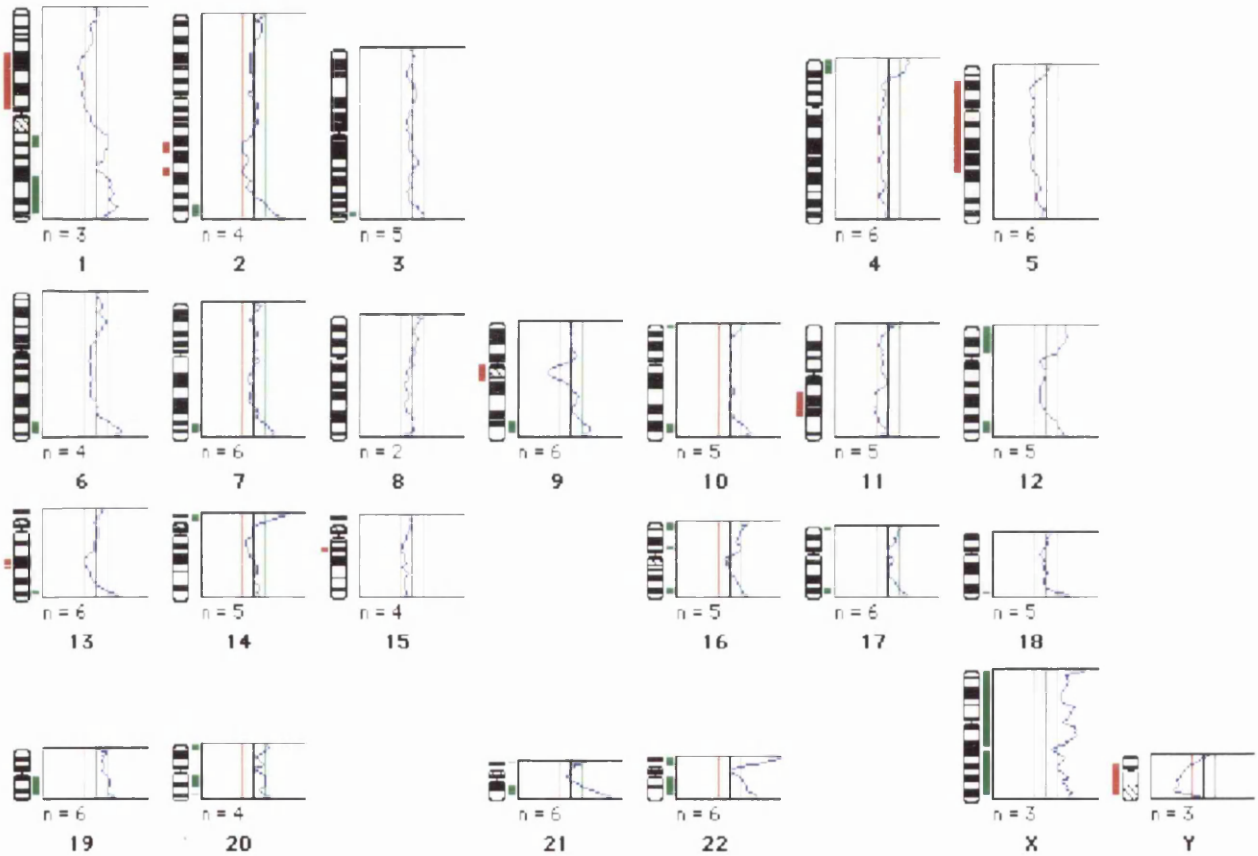


FIG 3.5.2-H CGH ratio profile using product amplified by DOP-PCR mehtod 6

The final work in optimizing the DOP-PCR involved a further comparison of enzymes in an attempt to improve the reproducibility and reliability of the results because microdissected tissue possessed only a very small cell mass. Pfu DNA polymerase, which possesses proofreading activity and thermostability, was applied. The fidelity of DNA synthesis by Pfu DNA polymerase is 12-fold higher than that of Taq DNA polymerase (Newton and Graham, 1994). CGH using this enzyme could not produce an analysable image at this time because further optimisation was required. Next, Expand High Fidelity DNA polymerase was tried on the samples of microdissected tumour cells from paraffin embedded melanoma tissue. Expand High Fidelity PCR System (EHF PCR System) is a mixture of thermostable Taq DNA polymerase and Pwo DNA polymerase. Pwo DNA polymerase has a half-life of 2 hours at 100°C, it is useful for high temperature primer extension. Due to the inherent 3′-5′ exonuclease proofreading activity of this enzyme, fidelity of DNA synthesis is 3-fold higher than that of Taq DNA polymerase. After processing optimization the EHF PCR system was used to analyse microdissected melanoma samples in parallel with very small amounts of DNA from three different sources as a positive control and results are presented in next part of this thesis.

Primer Extension Preamplification (PEP)

Primer Extension Preamplification (PEP) is an in vitro procedure developed to amplify a large fraction of the genome from limited amounts of DNA. Primer is composed of templates which are totally degenerated and fifteen nucleotides long. The template is first denatured at 92°C, and then primers are allowed to anneal at a low stringency temperature (37°C). The reliability of whole genome amplification by PEP has not yet been established and may prove difficult to evaluate in a few microdissected formalin fixed and paraffin-embedded solid tumour cells. It is possible that unequal allelic amplification may take place as a result of the small amount of template DNA derived from the small number of cells (Zhang et al., 1992; Faulkner et al., 1998). It is also possible for incorrectly amplified sequences to be produced due to the intrinsic error rate of Taq polymerase and for deletion mutations to be produced due to secondary structures of the template DNA (Dietmaier et al., 1999).
The aim of this experiment was to assess if whole genome amplification (WGA) using PEP could produce analysable CGH images and if so what

volume of starting template DNA could produce CGH images after WGA using PEP. Another aim was to evaluate some of the limitations and difficulties of using PEP and to assess the advantages and disadvantages in comparison with DOP-PCR.

Originally, protocol of PEP was developed by Zhang et al. (1992), and since then several modifications have been introduced into the basic protocols including changing the size or concentration of the primer, changing the number of cycles, the temperature or the enzyme used.

The PEP method of Faulkner et al. (1998) was used as the basic protocol in this experiment. This protocol was characterized by 1 in 10 primer concentration with 12-mer random oligonucleotides, minor changes of temperature and number of cycles (50 cycles to 40 cycles) in thermal cycling in comparison with the original PEP protocol by Zhang et al (1992). The modifications during the PEP optimisation are shown in Table 3.5.6.

In PEP method 1, the starting DNA was obtained from tumour cells in a 1 x 7 μm thickness section of ovarian cancer sample. When the product was run on gel after amplification by PEP, it was found that most of the DNA remained within the well.

DNA samples (3 x 7 μm sections) subjected to 4 days proteinase K treatment (DNA isolation method 4) were used in PEP method 2. The DNA smear on agarose gel (1.2 %) extended from the well to very small fragment size(< 100 bp) and intensity was very faint (Fig 3.5.2-I). CGH failed to produce adequate images.

DNA from the same sample but extracted by different methods (DNA isolation method 9-1,2) were investigated in PEP method 3. The first DNA (9-1) was extracted with phenol/chloroform extraction, the other DNA (9-2) was extracted without this step. By observation of the subsequent agarose gel with PEP amplification products, the DNA with phenol/chloroform extraction still remained in the well, while the DNA without phenol/chloroform extraction showed a smear from 12000 bp to below 300 bp (Fig 3.5.2-J). Amplified product using PEP with DNA obtained by DNA extraction method 9-2 produced CGH images in agreement with the combination of sex chromosomes used and some abnormal findings such as gains on chromosome 1q, 12p and losses on chromosome 1p, 11, and 15, but with prominent heterochromatin-rich regions on chromosome 1, 9 (Fig 3.5.2-K).

Table 3.5.6 Summary of PEP modification protocol

No	Amount of DNA (No.of Table 3.5.2)	Primer		$MgCl_2$	Phenol/ chlor. Extract.	Enz.	Conc.of DNA after PEP
		12mer (conc.)	15mer (conc.)				
1	10μl,1x7μm (No.2)		200pmol	5mM	+	AmpliTaq polymerase	
2	10μl,3x7μm (No.4)	200pmol		5mM	+	AmpliTaq polymerase	450ng/μl
3	10μl,1x7μm (No.9-1,2)	200pmol		5mM	–	AmpliTaq polymerase	340ng//μl
4	10μl,1x7μm (No.10-1)	200pmol		5mM	–	AmpliTaq LD	600ng/μl
5	10μl,1x7μm (No.10-2)	200pmol		5mM	–	AmpliTaq polymerase	510ng/μl
6	10μl,1x7μm (No.10-1)	200pmol		5mM	+	AmpliTaq LD	460ng/μl
7	10μl,1x7μm (No.10-2)	200pmol		5mM	+	AmpliTaq polymerase	44ng/μl
8	10μl,1x7μm (No.11-1)	200pmol		5mM	–	AmpliTaq LD	710ng/μl
9	10μl,1x7μm (No.11-2)	200pmol		5mM	–	AmpliTaq LD	750ng/μl
10	10μl,1x7μm (No.11-3)	200pmol		5mM	–	AmpliTaq LD	540ng/μl
11	10μl,1x7μm 11-1 11-2	200pmol		5mM	–	AmpliTaq LD	400ng/μl 490ng/μl
12	10μl,1x7μm (No.12)	200pmol		2.5m M	–	AmpliTaq LD	
13	10μl,1x7μm (No.13)	200pmol		5mM	–	AmpliTaq LD	510ng/μl
			200pmol	5mM			490ng/μl
14	10μl,1x3μm	200pmol		5mM	–	AmpliTaq LD	
15	10μl,1x7μm		800pmol	5mM	–	EHF PCR system	

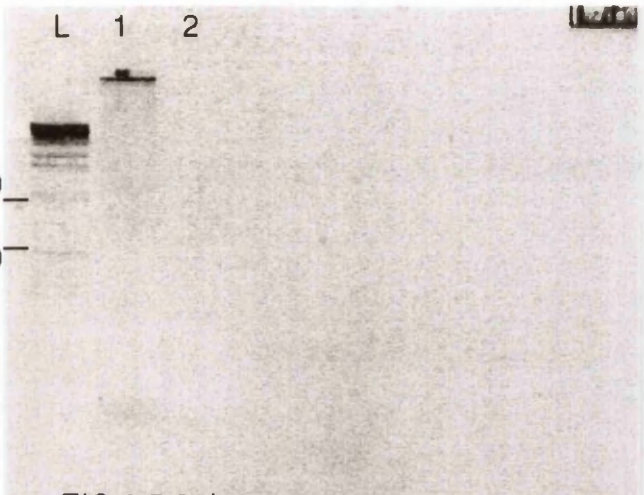


FIG 3.5.2- I
Gel electrophoresis of PEP product amplified by method 2
L; 1 kb ladder
1; PEP method - 2
2; negative control

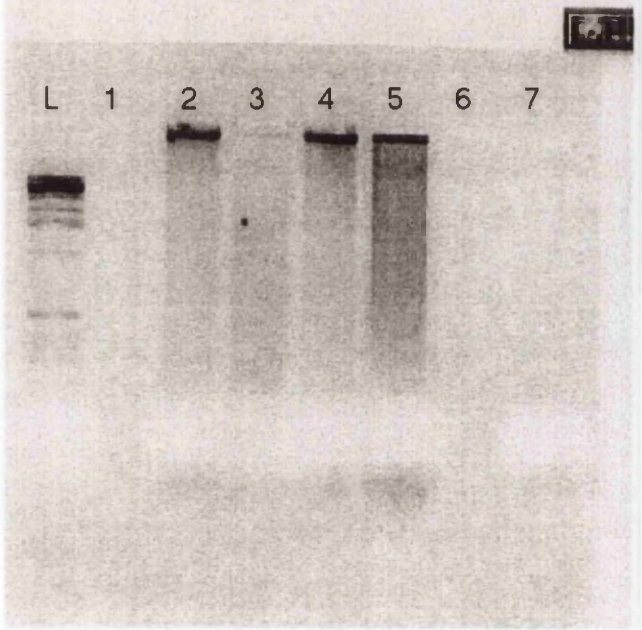
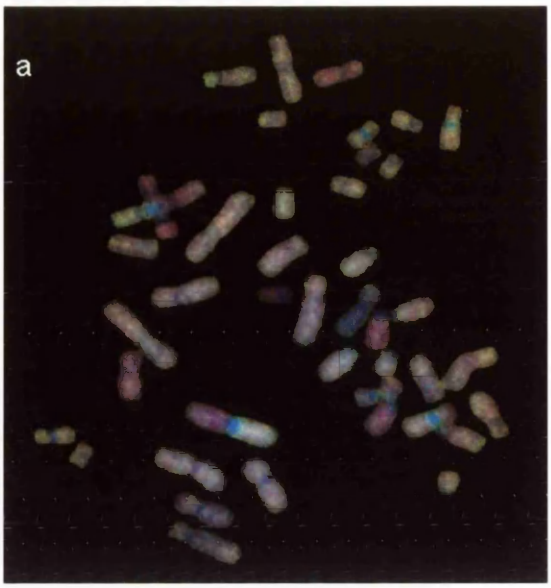


FIG 3.5.2- J
Gel electrophoresis of PEP products amplified by method 3 (9-1, 9-2)

L; 1 kb ladder
2; PEP method - 3 (DNA sample 9-1)
3; PEP method - 3 (DNA sample 9-2)
7; negative control



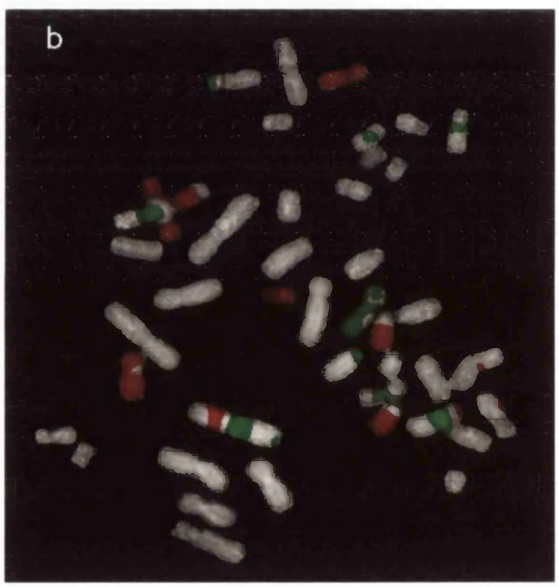


FIG 3.5.2-K a) CGH image following colour equalisation and b) colour ratio image using product amplified by PEP method 3 (9-2).

Methods 4, 5, 6 and 7 compared the results of PEP performed with or without phenol/chloroform extraction and using different enzymes. The results of gel running showed a wide spread from the well to very small fragmented DNA with faint intensity. Only method 4, which used AmpliTaq LD instead of AmpliTaq polymerase without phenol/chloroform extraction, produced rather improved CGH image. CGH showed true abnormal gains (chromosome 1q and 12p) and losses of DNA copy numbers (chromosome 5, 11, and 15) with disproportionately prominent centromeric region of chromosome 1 and 9, and the short arms of the acrocentric chromosomes but not accompanied with under or over-represented regions on chromosome 1p32-pter, 16, 19, and 22. The results indicated that phenol/chloroform probably decreases the yield of the PEP amplification to generate successful CGH. This was thought to be caused by the fact that the tissue sample might be too small to extract by the two-step standard method and remaining phenol might be having an inhibitory effect on PCR. Thereafter it was possible to make a decision to proceed with further experiments without phenol/chloroform extraction, and to use AmpliTaq DNA polymerase LD instead of AmpliTaq DNA polymerase.

In PEP methods 8, 9, and 10, template DNAs were produced by a slightly different method as described in experiments 11-1, 2, and 3 of previous section on DNA isolation methods. Methods 8 and 9 showed a very good size of DNA smear after PEP. From the point of view of CGH result, method 9 showed a better result but the intensity of green signal was still too weak to make analysable images but the finding of prominent centromeric regions on chromosome 1, 9, and the short arm of acrocentric chromosomes was not specific in comparison with previous results. The intensity of signal in method 8 was weaker than that of method 9 and was inadequate for analysis.

Method 10 failed to produce a CGH result.

In method 11, more complex strategies were applied to the same DNA sample as method 3 (DNA isolation method 9-2);

Sample 1; only PEP

Sample 2; PEP + DOP-PCR

After nick translation, the sample with PEP only showed a very faint but even smear, but the sample with PEP + another cycle of second DOP-PCR showed clear specific bands on the agarose gel as in the previous results of some DOP-PCR products (Fig 3.5.2-L). Comparing both CGH results, there were some discrepancies between these two results. Sample1 showed the first reliable,

homogeneous CGH image obtained until now, which could distinguish sex chromosomes and abnormal findings as in the previous experiments. No disproportionately over- or under-represented regions were seen except slightly prominent centromeric regions of chromosome 1 and 9 (Fig 3.5.2-M). Sample 2 showed matched sex chromosomes and some abnormal findings but also under-represented G-band regions and over–represented chromosome 19, 22 and some telomeric regions (Fig 3.5.2-N). CGH result with sample 2 was similar to the CGH findings with pure DOP-PCR product. Even though both CGH experiments used the same amount of Cot-1 DNA for suppression, over or under-represented regions were different between the two methods.

In methods 12 and 13, a better result was obtained from 5mM $MgCl_2$ with 12-mer primer, but the difference was not significant in the size of DNA fragments and the quality of CGH was also similar. To recognize the difference of amplification method between PEP and DOP-PCR, DOP-PCR was applied to the same DNA as these PEP experiments. CGH results using PEP amplification products showed very homogeneous, uniform images with constant abnormal findings in comparison with previous experiments, and were accompanied with disproportionately prominent green on heterochromatin-rich area such as centromeric regions on chromosome 1, 9, 16, and the short arms of the acrocentric chromosomes. CGH results using DOP-PCR amplification products showed a less homogeneous, granular pattern with the same abnormal findings but were less distinctive than those for PEP and some telomeric regions, and chromosomes 19 and 22 were over-represented. Interestingly, the centromeric regions on chromosomes 1, 9, and 16 appeared well suppressed even when the same amount of Cot-1 was used for blocking middle and highly repetitive DNA sequences (Fig 3.5.2-O).

Overall, the difference between CGH results by PEP and that by DOP-PCR was recognizable. More homogeneous, better quality images were obtained by PEP rather than by DOP-PCR above certain amounts of template DNA. PEP tended to amplify heterochromatin-rich regions; DOP-PCR tended to amplify some telomeric regions (G-light band areas) and GC-rich areas. The concentration of template DNA was critical in the production of reliably amplified product.

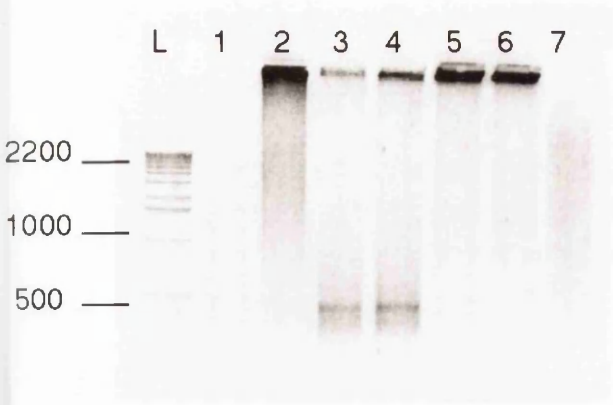


FIG 3.5.2- L

Gel electrophoresis of PEP products amplified by method 11

L; 1 kb ladder
1; negative control
2; PEP method - 11(sample 1)
3,4; PEP method -11 (sample 2)

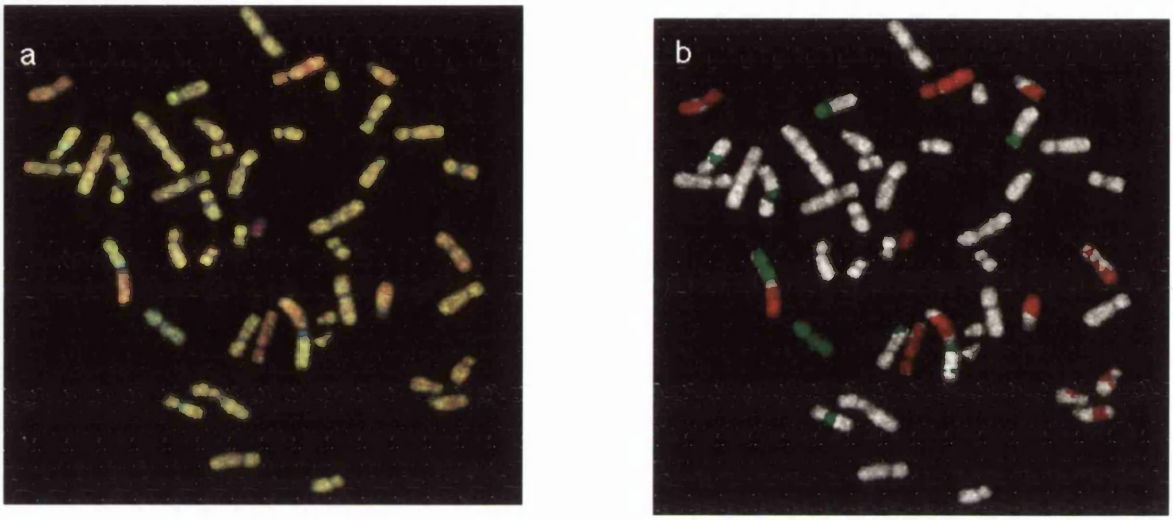


Fig 3.5.2-M a) CGH image following colour (fluorescence) equalisation and b) colour ratio image using product amplified by PEP method 11-1

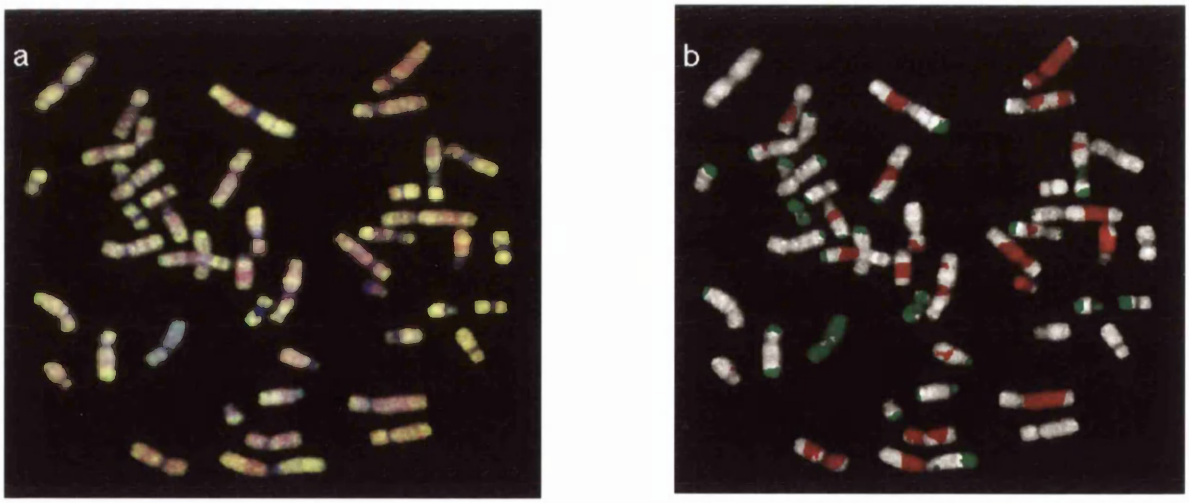


Fig 3.5.2-N a) CGH image following colour (fluorescence) equalisation and b) colour ratio image using product amplified by PEP method 11-2

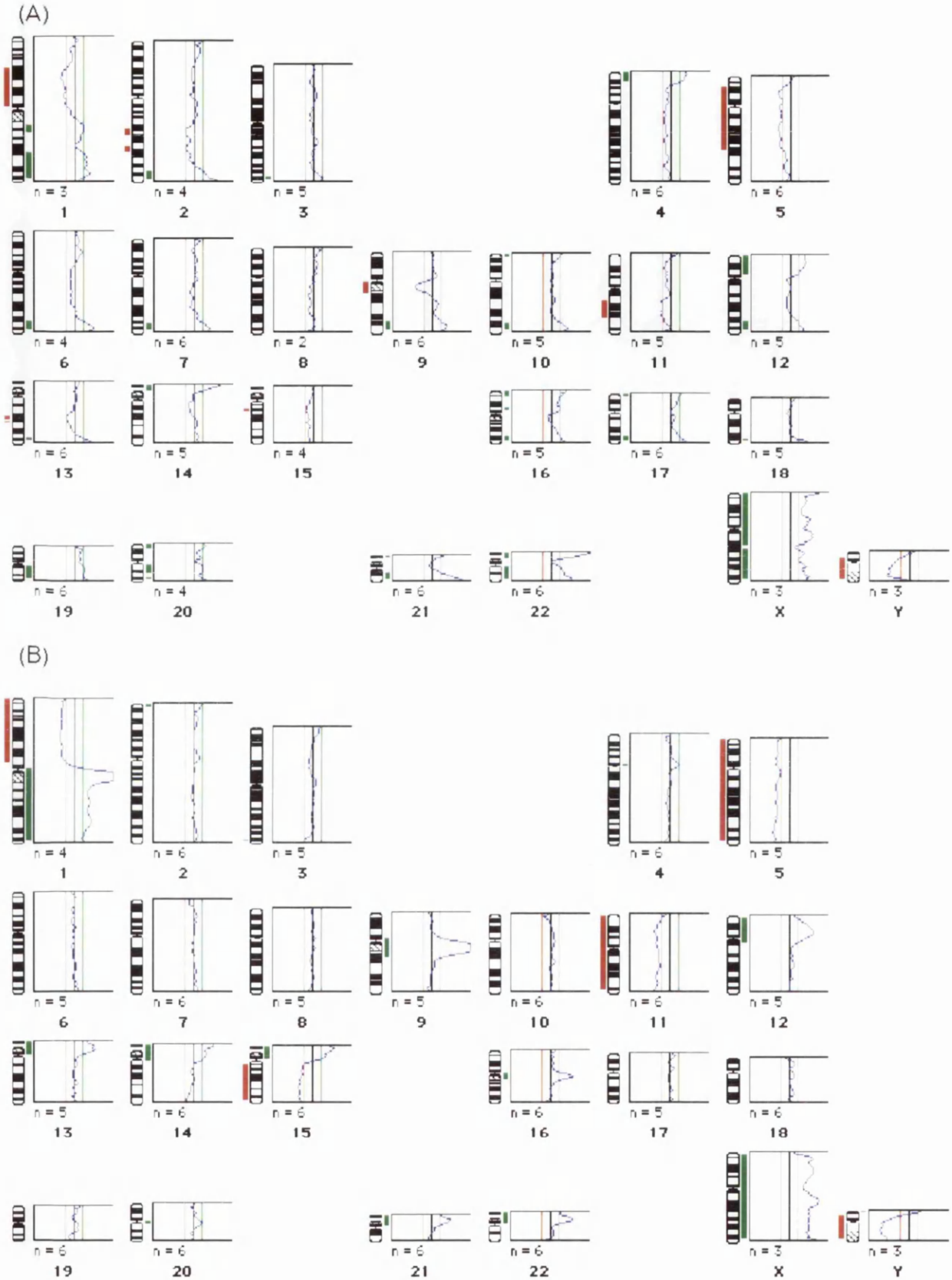


Fig 3.5.2-O CGH ratio profiles of two amplification methods using the same DNA. (A) CGH ratio profile using product by DOP-PCR amplification (B) CGH ratio profile using product by PEP amplification

After that, PEP was changed to the schedule of thermal cycling reported by Dietmaier et al (1999) and the EHF PCR system was introduced to the experiment to increase efficiency and fidelity of PEP amplification. Template DNAs were obtained from many melanoma samples, serially diluted DNA from normal female blood, abnormal bone marrow and thick section of paraffin-embedded ovarian cancer tissue. CGH results using these amplified products produced hardly any successful results.

The most troublesome problem during PEP amplification was that the negative control frequently showed a positive smear on agarose gel. The source of contamination was difficult to find because all reagents except primer and equipment were the same as for DOP-PCR which had not shown any contamination at all. CGH of amplified negative control which had shown a positive smear on the gel revealed no gain or loss of DNA copy number, and repeated CGH of amplified test DNAs from the same sample showed consistent results.

3.5.3 Labelling the amplified probe

The amplified genomic DNA (test DNA or reference DNA) was directly labelled with FITC-conjugated dUTP or Texas-Red-conjugated dUTP by PCR or nick translation. This method has an advantage in the simplicity of detection and in the improvement of quality of hybridisation although the detection signal is slightly reduced in comparison with the indirect detection method. Enzymatic labelling of DNA from paraffin-embedded tissue by nick translation may result in production of fragments which are smaller than the ideal size for CGH. Although the fragmented probe length of 200-400 bp is suitable for in situ hybridisation, a longer probe length (500-2000 bp) is required for CGH.

In the first part of this experiment, two modifications of nick translation were investigated to try to achieve longer size of probe, including adjusting the ratio of DNase 1 to DNA polymerase 1 enzymes in the reaction mixture and/or the incubation time. The length of DNA fragments after nick translation was thought not to be seriously affected by changing these parameters.

Later the labelling method was changed to incorporation during PCR amplification. The PCR labelling procedure was carried out during the second or the third rounds of PCR. The problem was that it was very difficult to estimate the labelling efficiency of both labelling methods especially the

PCR labelling procedure. Incorporation of fluorochrome-labelled nucleotides can be checked by placing a small amount of probe on a glass slide and examining for fluorescence using an epifluorescence microscope with appropriate filters or by rapid labelling assay. Labelling efficiency was roughly estimated by these methods. When PCR-labelled test DNA was hybridized with nick translated non-amplified reference DNA, it was very complicated to estimate the required amount of DNA from each sample because the two labelling methods showed different efficiency in the incorporation of fluorochromes (FITC or Texas-Red conjugated dUTP) into the DNAs.

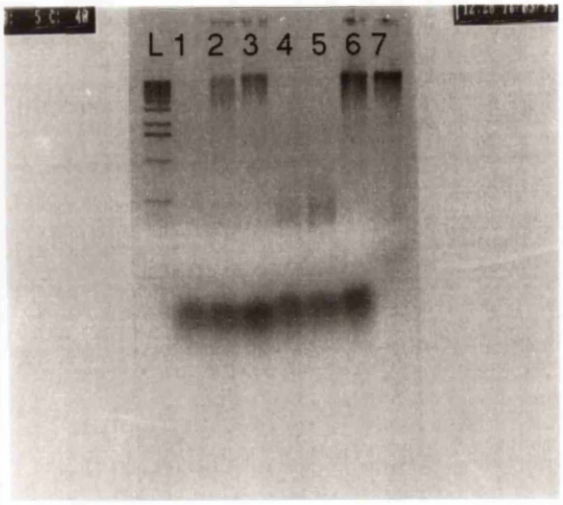
Labelling of DOP-PCR products

Comparing probe labelled by nick translation and by PCR, the size of DNA labelled by DOP-PCR was slightly longer than that by nick translation. The summary of results depending on the DOP-PCR method is listed in Table 3.5.7. Smears in agarose gels of each case are shown in Fig 3.5.3-A.
From the data in Table 3.5.7 it also is clear that obtaining the optimum size of probe depends not only on the labelling method but also on the quality and quantity of starting DNA. The latter appears to be more critical than any of the other parameters investigated.

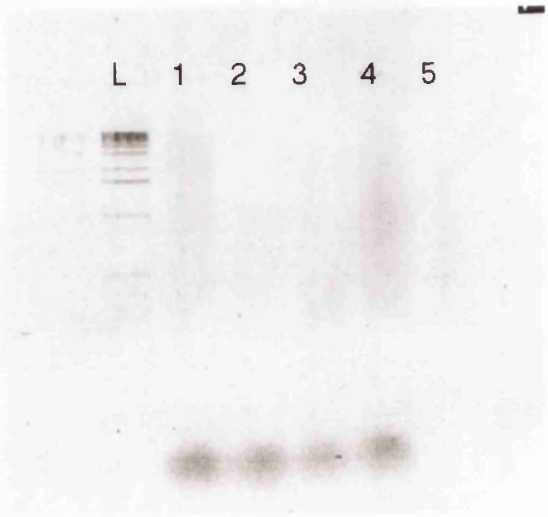
Table 3.5.7 Summary of the gel running results comparing PCR labelling with nick translation

No.(same as Table 3.5.4)	Labelling during third PCR	Nick translation	Probe size after gel running
1	–	+	< 500 bp
2	–	+	< 500 bp
3	–	+	< 500 bp
4	–	+	200- 1000 bp
5	+	–	300- 1000 bp
6	+	–	300- 500 bp

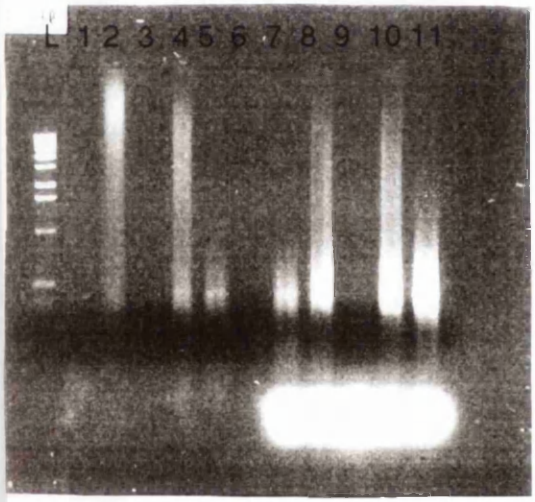
FIG 3.5.3-A Gel electrophoresis of the DOP-PCR products labelled by nick translation and DOP-PCR labelling



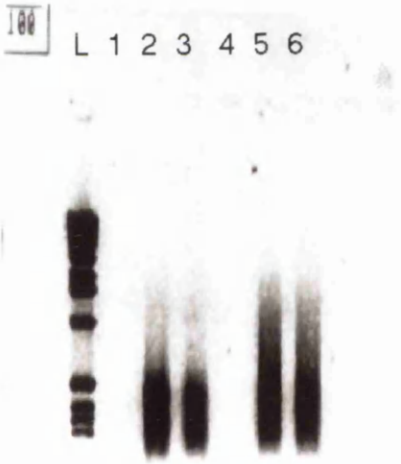
L; 1 kb ladder
4; DOP-PCR method - 1 after nick translation
5; DOP-PCR method - 2 after nick translation



L; 1 kb ladder
2; DOP-PCR method - 4 after nick translation
3; DOP-PCR method - 3 after nick translation



L; 1 kb ladder
8; DOP-PCR method - 5 after PCR labelling



L; 1 kb ladder
2,3; DOP-PCR method - 6 after PCR labelling

Labelling of PEP product

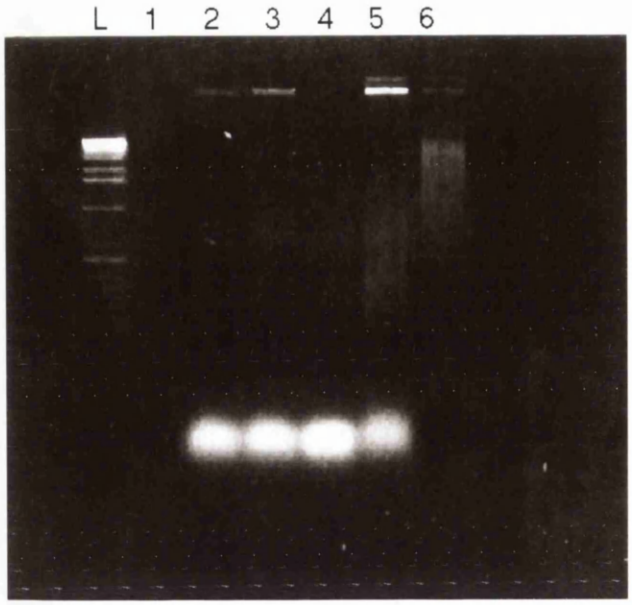
All PEP products were labelled by nick translation. When run on a gel, the nick translated DNA was extremely faint, but the smear of DNA fragments on agarose gel showed mostly from 1000 bp to 200 bp with some remaining near the well in some PEP experiments (Fig 3.5.3-B). Sometimes the size of probe was longer than that obtained by DOP-PCR labelling, but in these instances there was insufficient product for CGH. The difference of efficiency of amplification between the two methods was thought to be affecting the success rate for CGH.

Problems encountered after the labelling procedure

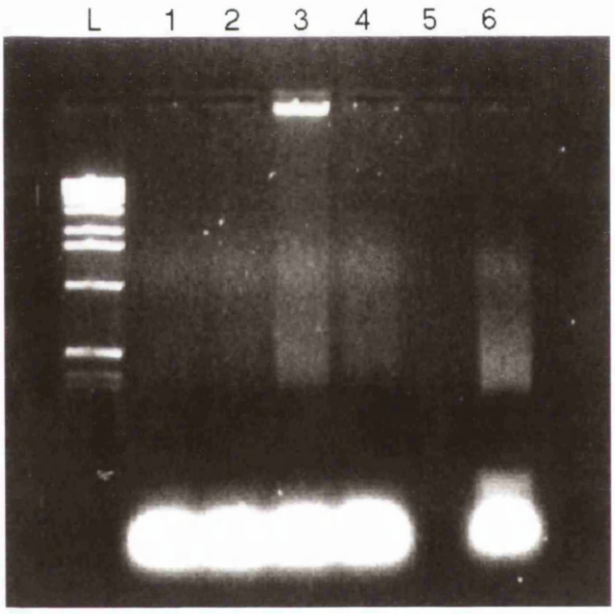
One of the major problems associated with labelling procedures is the question of what proportion of probe (test and control DNA) has been labelled by both labelling methods. Consequently, unbalanced amounts of DNA between test and control DNA induced false positive or false negative results and unmatched sex chromosomes. After several failures of CGH, these problems were somewhat resolved by using hybridization with simultaneously amplified and labelled reference DNA by same method. But differences of length of fragments (complexity) between test DNA (usually from paraffin embedded tissue) and reference DNA (from fresh normal male and female blood) caused difficulties in obtaining good quality of CGH images due to different competition effects during hybridisation.

To optimise the amount to be hybridised after labelling procedure, two different methods were applied to the amplified product to estimate the percentage of DNA labelled with fluorochrome. First, amplified and labelled product was purified using High Pure PCR Product Purification Kit (Boehringer Mannheim). The DNA concentration before and after purification was estimated by measuring optical density (O.D.) at 260 nm in a spectrophotometer. The percentage of DNA labelled with fluorochrome was calculated by the fact that this purification kit can remove salts, proteins, dimerized primer, and unincorporated nucleotides. Examples of the results are shown in Table 3.5.8 and 3.5.9.

FIG 3.5.3-B Gel electrophoresis of the PEP products labelled by nick translation



L; 1 kb ladder
2; PEP method - 2 after nick translation
3; PEP method - 3 after nick translation



L; 1 kb ladder
1; PEP method - 4 after nick translation
2; PEP method - 5 after nick translation
3; PEP method - 6 after nick translation

Table 3.5.8 DNA concentration before and after purification of Nick translation products after PEP

Case number	DNA conc. before purification (/μl)	DNA conc. after purification (/μl)	Percentage of DNA with fluorochrome incorporated
1	280 ng	130ng	46.4%
2	250ng	110ng	44%
3	390ng	170ng	43.6%
4	300ng	120ng	40%
5	380ng	140ng	36.8%

Table 3.5.9 DNA concentration before and after purification of PCR labelling products after DOP-PCR

Case number	DNA conc. before purification (/μl)	DNA conc. after purification (/μl)	Percentage of DNA with fluorochrome incorporated
1	660 ng	40ng	6%
2	750ng	65ng	8.6%
3	710ng	80ng	11.3%
4	860ng	95ng	11%
5	900ng	65ng	7.2%

By observation of these results, it was roughly estimated that 6 to 11% of DOP-PCR product was labelled during DOP-PCR labelling, 36 to 46% of PEP product was labelled during nick translation.

The second method was semi-quantitative labelling assay which was designed based on the fluorescent properties of the label. In this rapid labelling assay, the labelled probe was dotted onto a membrane (hybond-N+ nylon), then membrane washed for 20 min at 60°C to remove unincorporated nucleotide and the bound probe was visualized by a U.V. transilluminator. When compared with diluted original fluorochrome (100%, 75%, 50%, 25%, 12.5%, 6.25%), DOP-PCR labelling efficiency was approximately from 6.25% to 12.5% by rough estimation. This result was compatible with that of purification kit method.

After rough estimation of the concentration of labelled DNAs, the next step was to optimise the amount of test DNA per reference DNA to be hybridised. When the hybridisation with same amount of test and reference DNA was applied to a CGH experiment, the CGH result always showed less intensity of green than of red. Because the amount of optimally sized DNA fragments in test DNA was less than that in the control DNA, competitive binding of test DNA during

hybridisation might be less effective. In this experiment, when 2-2.5 fold of test DNA was hybridised with reference DNA the CGH result revealed mostly analysable images.

3.5.4 CGH Experiments with serially diluted DNA samples

The aim of the next part of the work was to evaluate the limiting amount of initial test DNA (template DNA) required to produce analysable CGH images from the various types of sample and to provide standards of validation to results of unknown samples as a positive control.
The basic DNA sources for these experiments were DNA from normal female blood (initial conc.: 800ng/μl), DNA from abnormal bone marrow with AML/ MDS (initial conc.: 1μg/μl), DNA from abnormal paraffin-embedded ovarian cancer tissue (initial DNA conc.: 1μg/μl).
Samples were diluted carefully inside the containment hood to avoid contamination. A serial dilution of each sample was conducted on the basis of an amount of template DNA in 50 μl of the first PCR buffer.
Primer-Extension Pre-amplification (PEP) and DOP-PCR as a method of whole genome amplification were performed on each sample.
CGH was performed with three control samples along with DNA from test samples of unknown karyotype.

Three different strategies were applied to each DNA sample.

1) Amplified test DNA was hybridised with non-amplified, nick-translated control DNA.
2) Amplified test DNA was hybridised with amplified control DNA (Starting concentration was the same.).
3) Different kinds of enzyme were used during amplification.

Ampli Taq DNA Polymerase
Pfu DNA Polymerase
Expand High Fidelity DNA Polymerase;
enzyme mix containing thermostable Taq DNA and Pwo DNA polymerase

DNA from Normal Female Blood

Table 3.5.10 Schematized strategy applied on DNA from normal female blood

Basic source of DNA	Amplification method	Amount of template DNA	CGH result	Enzyme used
Normal female blood	Hybridisation Method; amplified test DNA with non-amplified Nick-translated control DNA			
	PEP	40 ng	fail	AmpliTaq LD
		4 ng	fail	AmpliTaq LD
		2 ng	fail	AmpliTaq LD
	PEP	40 ng	inadeq-uate	EHF PCR system
	DOP-PCR	12 ng	good	Thermosequenase
		1.2 ng	good	Thermosequenase
		600 pg	good	Thermosequenase
		300 pg	good	Thermosequenase
		150 pg	good	Thermosequenase
		75 pg	good	Thermosequenase
		50 pg	fail	Thermosequenase
		25 pg	fail	Thermosequenase
Normal female blood (test DNA) Normal male blood (control DNA)	Hybridisation Method; amplified test DNA with amplified control DNA			
	PEP	40 ng	good	EHF PCR system
	DOP-PCR	100 pg	good	AmpliTaq LD &
		50 pg	good	EHF PCR system
		10 pg	good	

1) Amplified test DNA (normal female) hybridised with non-amplified, nick-translated DNA

Amplification Method; PEP

Both the amplified test DNA and the non-amplified control DNA were labelled by nick translation. Before nick translation, DNA concentration of each amplified sample was checked by measuring the optical density with a spectrophotometer and the amount of test DNA compared with reference DNA required for

hybridisation was calculated.

After PEP amplification with 40 ng of template DNA, the DNA smear on ethidium bromide-stained agarose gel showed from 500 bp to high molecular weight DNA (mainly over 12000 bp) (Fig.3.5.4-A).

After labelling by nick translation, product amplified from 40 ng of normal female DNA was still mainly near the well on agarose gel.

In the case with amplification products from 40 ng of normal female template DNA using PEP with AmpliTaq LD, CGH showed a very weak intensity of green in comparison with the intensity of red. Prominent strong green showed on heterochromatin-rich area (centromere on chromosome 1, 9, 16, short arm of acrocentric chromosomes) and on some telomeric regions with a lot of non-specific background signals (Fig. 3.5.4-B).

In the case with amplification from 4 ng of template DNA, the intensity of the DNA smear was very faint with over 1000 bp sized DNA fragments (Fig 3.5.4-A). Several trials of CGH using PEP product amplified from 40 ng, 4ng, and 2 ng of normal female template DNA failed to produce successful CGH images.

Up to this point PEP had been performed using the enzyme AmpliTaq LD. Because of the repeated failure to achieve CGH results, the EHF PCR system was substituted, and amplified product from 500 to over 12000 bp with strong intensity was achieved (Fig. 3.5.4-C). After nick translation, the size of DNA fragments was from 300 bp to 1000 bp and subsequent CGH produced improved images (test DNA; labelled with Texas-Red, control DNA; labelled with FITC, hybridised on male metaphase) (Fig. 3.5.4-D).

Two different sizes of primer (12 mer, 15mer) were investigated for PEP, but no difference in the amplified product could be detected.

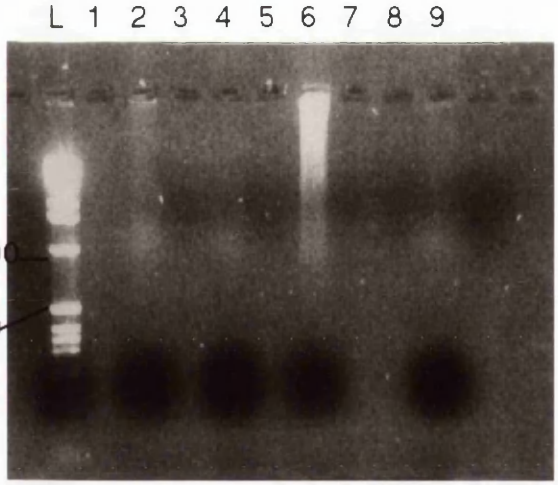
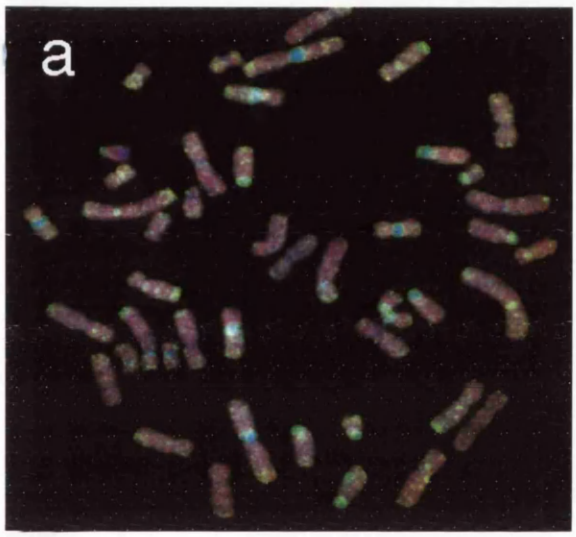
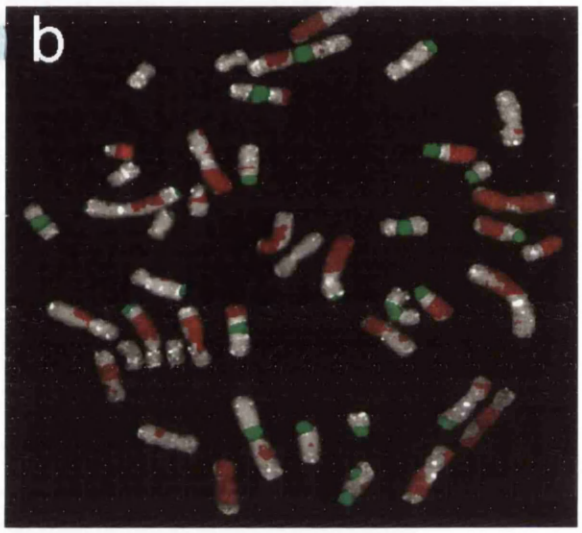


FIG 3.5.4- A

Gel electrophoresis of the PEP products using AmpliTaq LD

L; 1 kb ladder
2; 4 ng of normal female DNA
6; 40 ng of normal female DNA
9; negative control





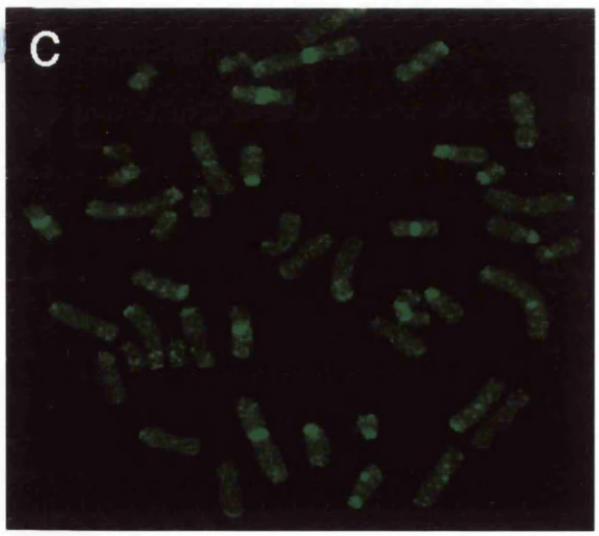


Fig 3.5.4-B (a)CGH image following colour equalisation (test DNA; 40 ng of template DNA from normal female blood amplified by PEP using AmpliTaq LD and labelled FITC, control DNA; non-amplified, nick translated normal male DNA and labelled with texas-red, and hybridised on normal male metaphases. (b) colour ratio image of same metaphase. (c) hybridisation pattern of FITC labelled test DNA of same metaphase after split colour cord.

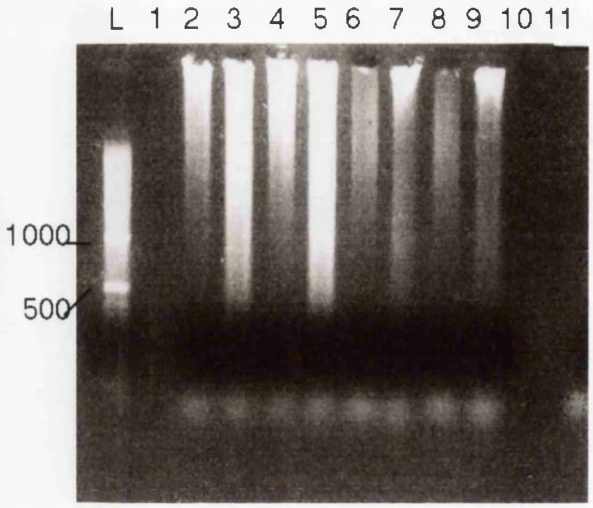
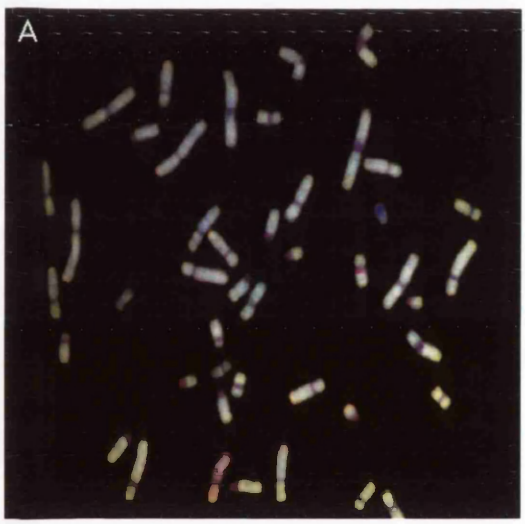


FIG 3.5.4 - C

Gel electrophoresis of the PEP product using EHF PCR system (EHF) and Expand long Template system (EL)

L; 1 kb ladder
2; case 5 (982963) with EHF
3; 40 ng of normal female DNA with EHF
7; 40 ng of normal female DNA with EL
11; negative control



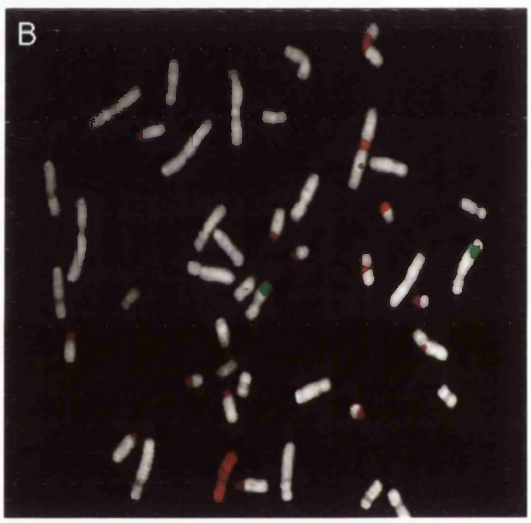


Fig 3.5.4-D (A) CGH image following colour equalisation (test DNA; 40 ng of normal female template DNA amplified by PEP with EHF PCR system and labelled with Texas-Red, control DNA; non-amplified, nick translated normal male DNA and labelled with FITC, hybridised on male metaphases. (B) colour ratio image of the same metaphase.

Amplification Method; DOP-PCR

Normal female DNA was amplified using DOP-PCR and the template DNA which had been diluted from 12 ng to 25 pg in 50 μl of PCR reaction mixture.

Amplified product from 12 ng of template DNA showed stronger intensity than that from paraffin-embedded ovarian cancer tissue (1x 7 μm) and amplified product from 1.2 ng showed a very faint smear on agarose gel (Fig 3.5.4-E). After PCR labelling, both normal female samples (12 ng, 1.2 ng) showed bigger DNA fragment sizes (300-1600 bp) than DNA from paraffin-embedded tissue of unknown test sample (below 500 bp).

DNA sizes of amplified product from 300 pg of normal female DNA was from 500 bp to over 12000 bp (not remaining in well), and the intensity was similar to that of amplified product from 1.2 ng of DNA from abnormal bone marrow. DNA smears of 150 pg of normal female DNA showed less intensity and similar sizes of DNA fragment (Fig.3.5.4-F).

At the level of 75pg, DNA smears after the first DOP-PCR amplification showed relatively ideal sizes of DNA (220-1600 bp) with moderate intensity (Fig 3.5.4-G).

Below the concentration of 75 pg further dilutions of template DNA yielded after amplification smears which were barely visible on the gel (Fig 3.5.4-G). However even if the DNA smear after first DOP-PCR was very weak on running the gel, CGH could sometimes be successful after the second amplification with labelling.

Thermosequenase was used as an enzyme in the first low annealing temperature cycles in this part of the work.

In CGH experiments of this part of the work all diluted samples were labelled by DOP-PCR labelling, and hybridised with non-amplified, nick-translated control DNA (normal male DNA) on male metaphases. CGH results of each diluted sample showed within normal range of fluorescence intensity ratio when threshold was decreased from 1.5/0.5 to 1.35/0.65. However these results revealed a tendency to under-representation on some chromosomal regions such as 1p32-pter, 16p, 17 (?), 19, and 22. After reaching the threshold 1.20/0.80, the latter findings became more obvious. Actually since these test samples were not contaminated with any other cells, it may be reasonable to apply a threshold of 1.5/0.5.

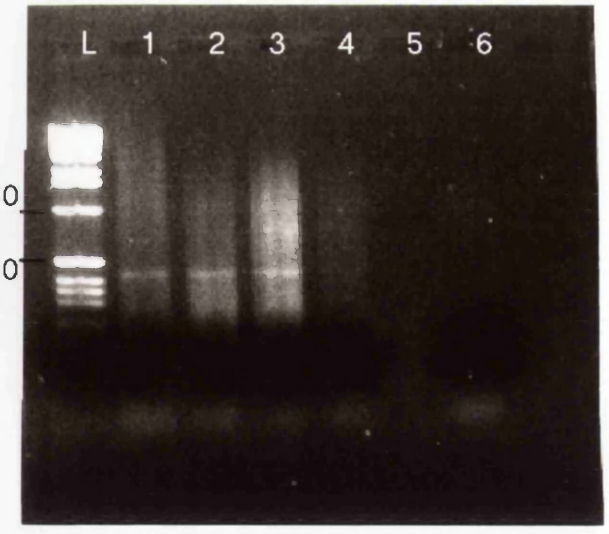


FIG 3.5.4 - E

Gel electrophoesis of the first DOP-PCR products using thermosequenase

L; 1 kb ladder
1; 1 x 7 um paraffin-embedded ovarian cancer tissue
3; 12 ng of normal female DNA
4; 1.2 ng of normal female DNA
6; negative control

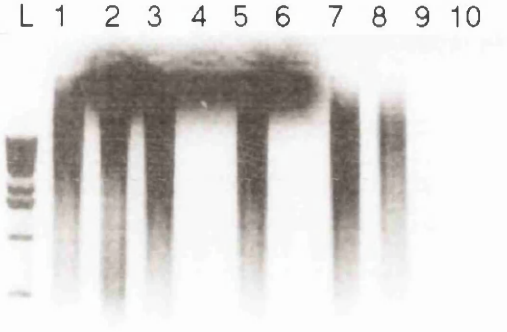


FIG 3.5.4 - F

Gel electrophoresis of the first DOP-PCR products using thermosquenase

L; 1 kb ladder
1; 1.2 ng of abnormal BM DNA
2; 600 pg of abnorma BM DNA
7; 300 pg of normal female DNA
8; 150 pg of normal female DNA
10; negative control

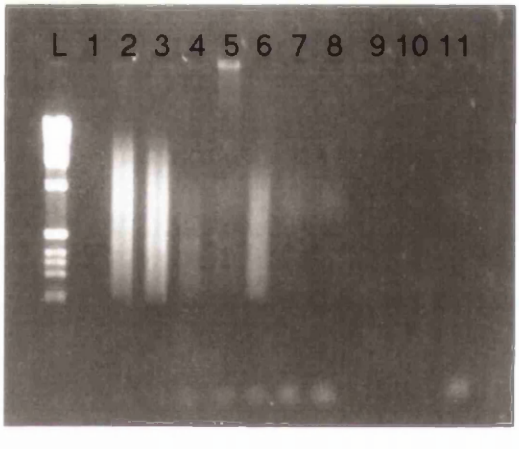


FIG 3.5.4 - G

Gel electrophoresis of the first DOP-PCR products using thermosequenase

L; 1 kb ladder
2; 300 pg of abnormal BM DNA
3; 150 pg of abnormal BM DNA
4; 300 pg of DNA from PET
5; 150 pg of DNA from PET
6; 75 pg of normal female DNA
7; 50 pg of normal female DNA
8; 25 pg of normal female DNA
11; negative control

In this experiment, it was difficult to know what percentage of fluorochrome has been incorporated during PCR labelling or nick translation. Then problems were induced by unbalanced amounts of labelled DNA between test and reference DNA because unbalanced amount of labelled DNAs may be too much for normalisation to take place.

From a literature review, even when the test DNA had not been amplified, in CGH results with indirectly labelled DNAs, GC-rich regions such as distal 1p, 16, 19, and 22 often show wide variability in the CGH ratio (Kallioniemi et al., 1994). The work presented here also showed a tendency to disproportionately under-representation on chromosome 1p33-pter, 16p, 17, 19 especially 19p and 22 until the amount of template DNA was above 75 pg. When the amount of template DNA was below 75 pg amplified product could not produce reliable CGH images. At very low concentrations of template DNA (50 pg, 25 pg), chromosomes distal 1p 19, 22 and some telomeric regions appeared unreasonably over-represented, where they were previously under-represented when the starting template DNA was above 75 pg. Dark band regions with G-banding showed much weaker green than other parts of the chromosomes. Chromosome 17 has not been highlighted as a target chromosome which showed a variable CGH ratio but in this study it frequently had a variable ratio.

2) Amplified test DNA hybridised with amplified control DNA

Amplification method: PEP

In this experiment, 40 ng of template DNAs (normal male and female) were amplified by PEP using EHF PCR system, then labelled by nick translation with two different fluorochromes (normal female DNA; labelled with Texas-Red, normal male DNA; labelled with FITC) and hybridised on normal male metaphases. The size of DNA fragments after amplification was very good with a strong intensity. The CGH result showed very uniform, reliable CGH images without any disproportionately over-or under-represented regions (Fig 3.5.4-H).

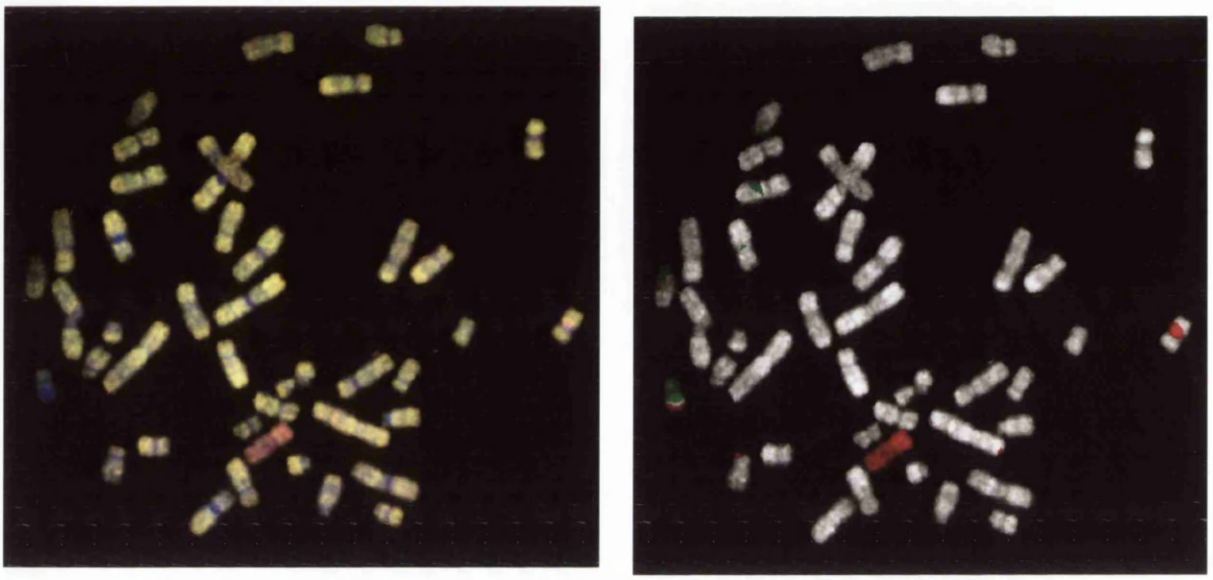


FIG 3.5.4-H (A) CGH image following colour equalisation (test DNA; 40 ng of normal female template DNA amplified by PEP with EHF PCR system and labelled with Texas-Red, control DNA; 40 ng of normal male template DNA amplified by PEP with EHF PCR system and labelled with FITC, hybridised on male metaphases. (B) colour ratio image of the same metaphase.

Amplification method: DOP-PCR

In this experiment, test DNA was obtained from normal female blood, reference DNA was from a normal male blood and the starting concentration for amplification using DOP-PCR was the same for both.

Because CGH using the previous strategy failed to produce a result when the amount of template DNA was below 75 pg, in this experiment template amounts of 100 pg, 50 pg and 10 pg were used.

AmpliTaq LD and EHF PCR system were used as enzymes during DOP-PCR amplification.

The intensity and size of DNA on agarose gel showed some differences among three different starting amounts of template DNA with AmpliTaq LD, on the other hand, products with EHF PCR system showed similar size and intensity of DNA fragments in addition to stronger and longer DNA fragments than those with AmpliTaq LD (Fig 3.5.4-I).

The CGH results with both enzymes showed quite uniform, evenly hybridised image and profiles (Fig 3.5.4-J). This experiment showed reliable CGH results without unreasonably over or under-represented regions on chromosome 1p32-pter, 19p, and 22. Sex chromosomes were also matched even at the 10 pg level and regardless of the enzyme used.

This experiment was started at the same concentration of both test and control DNAs, amplified by DOP-PCR simultaneously, hybridised with the same amount of test and control DNA. These conditions made it possible to avoid disproportionately over-represented or under-represented findings induced by mismatching of the amount between test and control DNA, different incorporation rate depending labelling methods, and bias from amplification process itself.

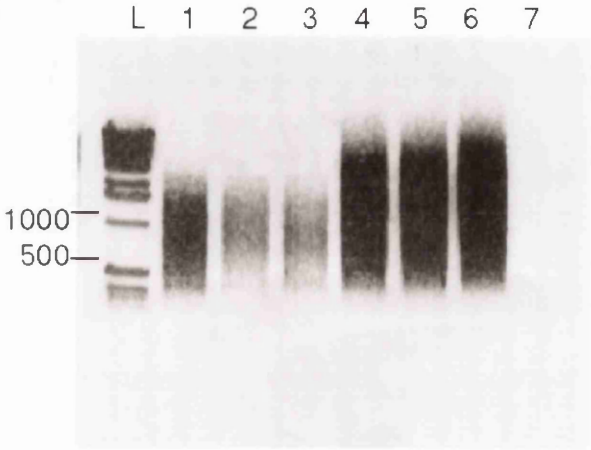


FIG 3.5.4 - I

Gel electrophoresis of the first DOP-PCR products using EHF PCR system (EHF) and AmpliTaq LD (A)
EHF PCR system; Expand High Fidelity PCR system

L; 1 kb ladder
1; 100 pg of normal female DNA with A
2; 50 pg of normal female DNA with A
3; 10 pg of normal female DNA with A
4; 100 pg of normal female DNA with EHF
5; 50 pg of normal female DNA with EHF
6; 10 pg of normal female DNA with EHF
7; negative control

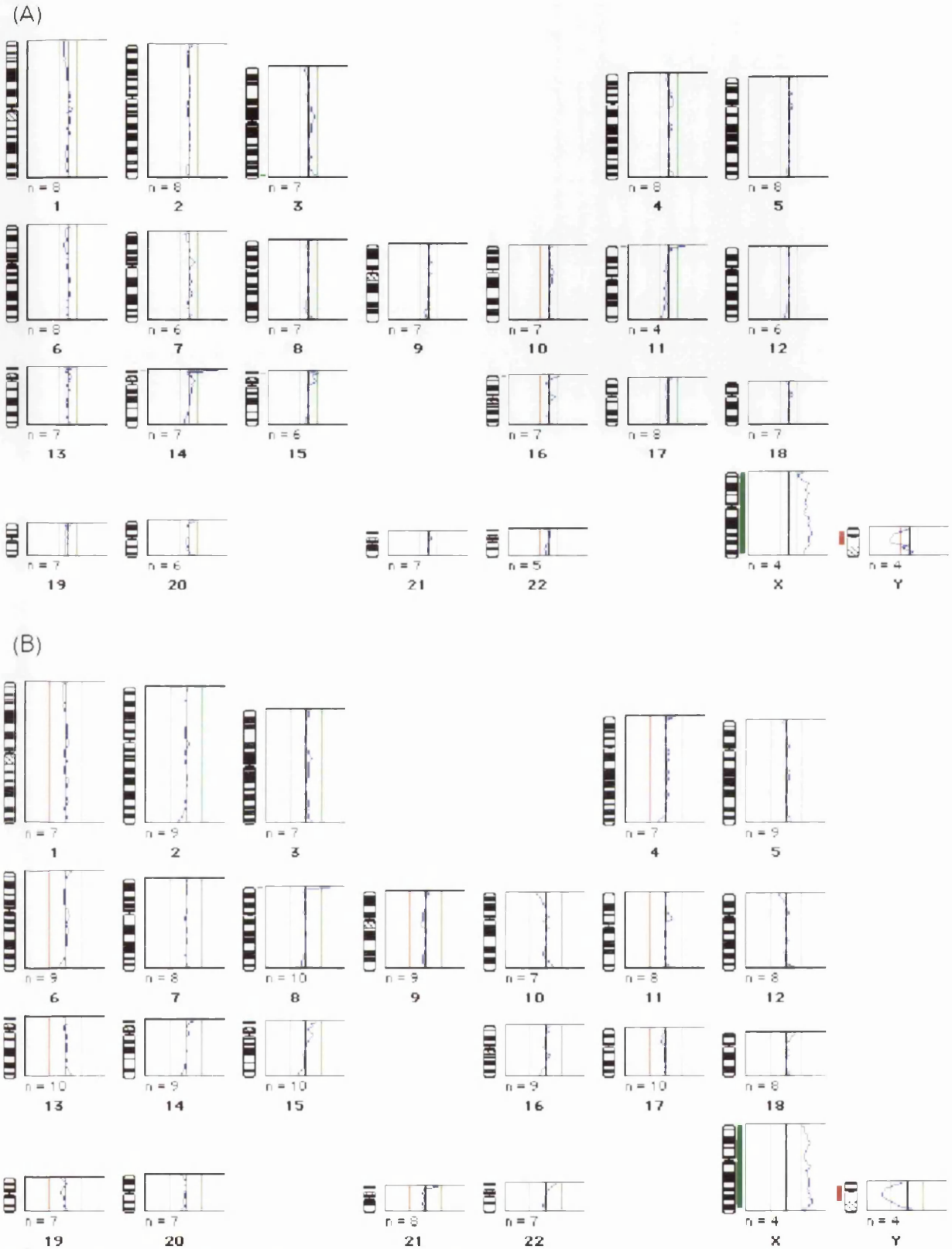


Fig 3.5.4-J CGH ratio profiles using products of DOP-PCR with 100pg of template DNA from normal female blood, hybridised the same amount of amplified normal male DNA. (A) DOP-PCR using AmpliTaq LD, (B) DOP-PCR using EHF PCR system

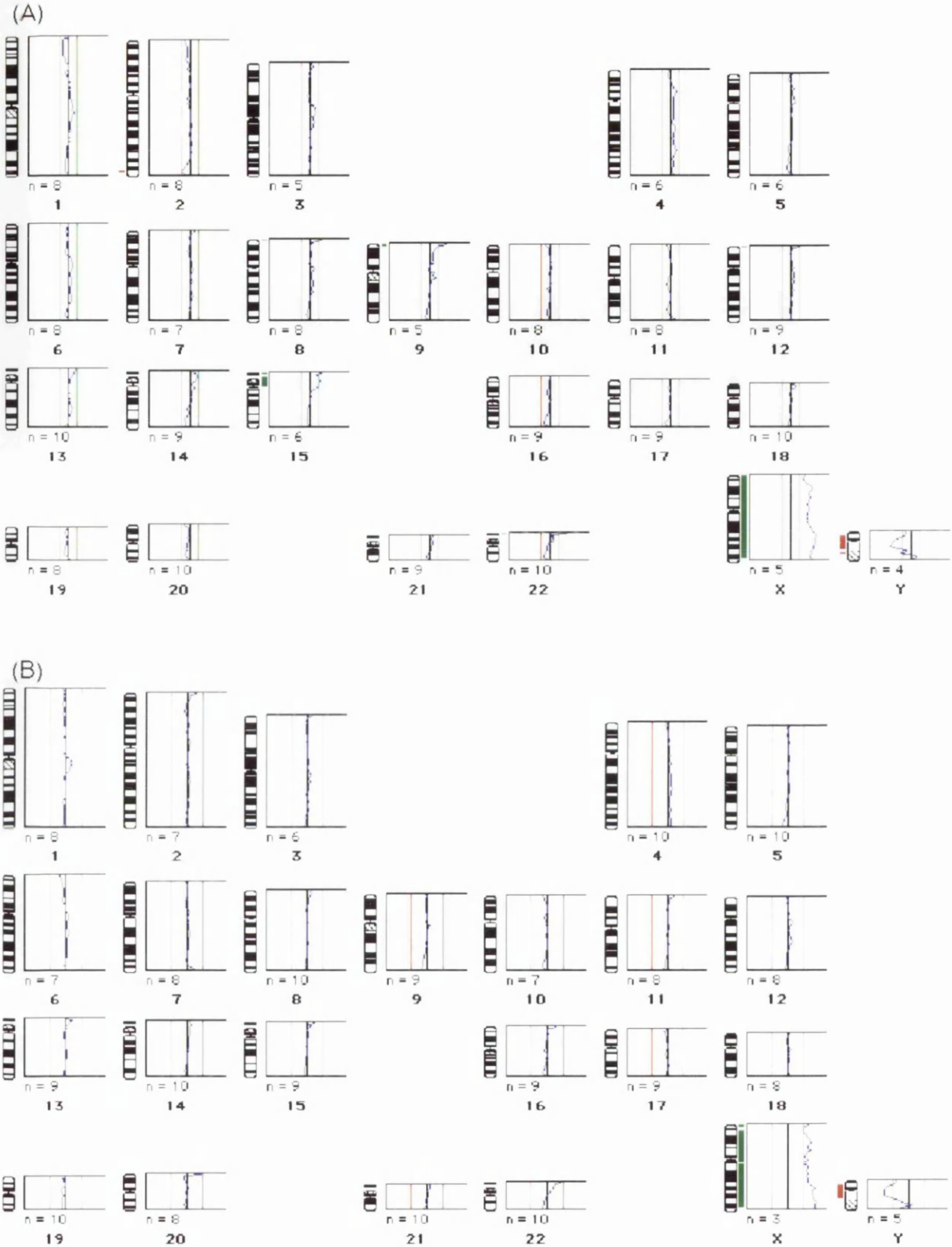


Fig 3.5.4-J CGH ratio profiles using products of DOP-PCR with 50pg of template DNA from normal female blood, hybridised with the same amount of normal male DNA. (A) DOP-PCR using AmpliTaq LD, (B) DOP-PCR using EHF PCR system

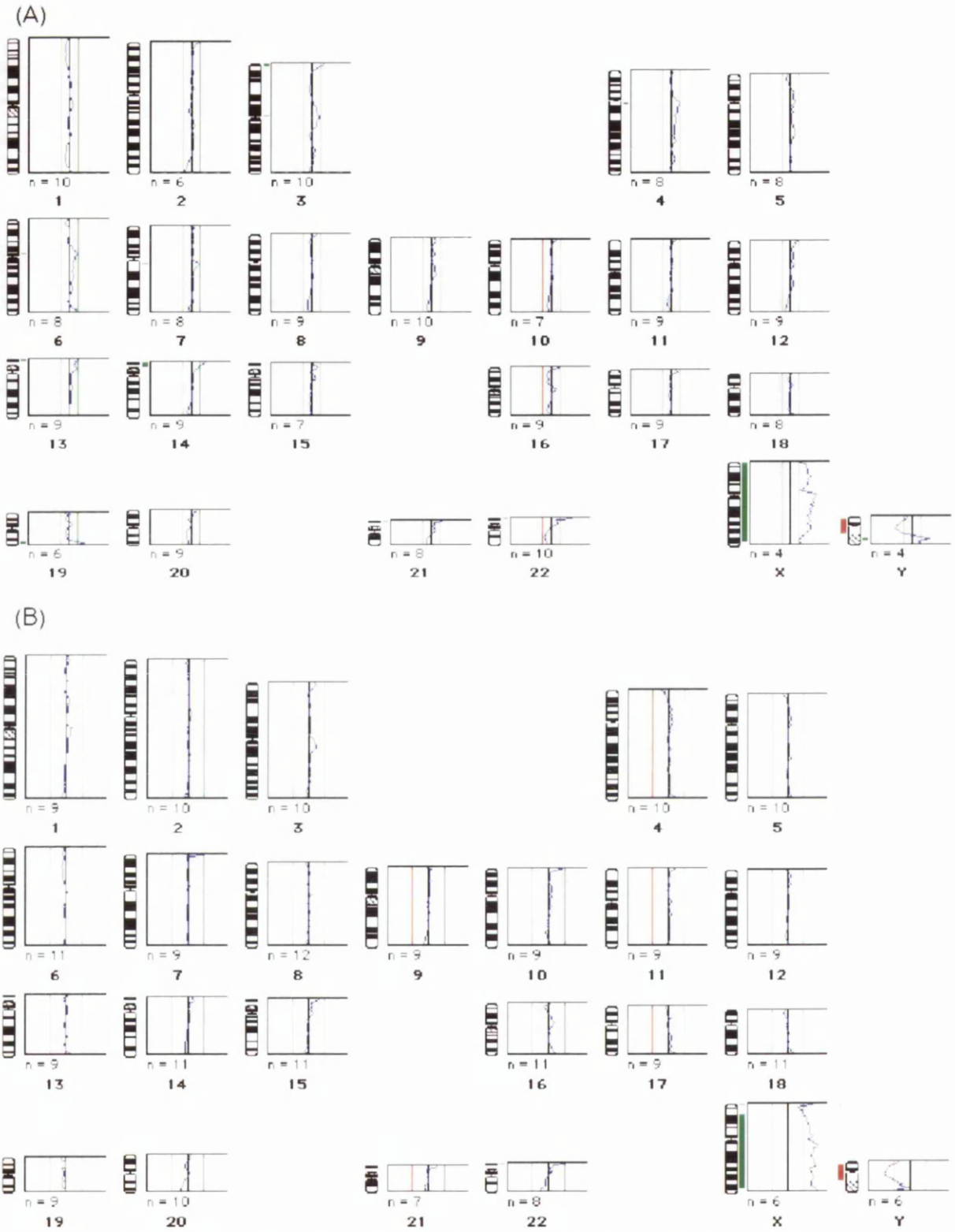


Fig 3.5.4-J CGH ratio profiles using products of DOP-PCR with 10pg of template DNA from normal female blood, hybridised with the same amount of normal male DNA. (A) DOP-PCR using AmpliTaq LD. (B) DOP-PCR using EHF PCR system.

3) Application of different kind of enzyme during PCR

Four different enzymes were investigated: thermosequenase, AmpliTaq LD, Pfu DNA polymerase, and Expand High Fidelity DNA polymerase on the course of this WGA using DOP-PCR and PEP.

By PEP amplification, only products using EHF PCR system could produce CGH images.

All samples hybridized with non-amplified control DNA were produced by DOP-PCR amplification with thermosequenase (DOP-PCR method 6). All showed relatively reasonable CGH results with good coverage of the whole 24 chromosomes with an under-represented tendency on chromosome 1p32-pter, 16, 17, 19, and 22. Then the schedule of thermal cycles in the DOP-PCR protocol was changed such as increasing number of lower annealing temperature cycles (4 cycles→ 7 cycles) and extension time (2 min→3 min) and was AmpliTaq LD used instead of thermosequenase. Although the concentration of starting DNA was different in each case, results using either thermosequenase or Ampli Taq LD did not show significant difference.

As the concentration of template DNA was decreased, a more reliable DOP-PCR method was required. Pfu DNA polymerase and EHF DNA polymerase were introduced to WGA during the DOP-PCR.

Pfu DNA polymerase failed to yield a proper result in these samples because of inadequate optimisation. EHF PCR system could be optimised by increasing the concentration of primer and $MgCl_2$.

Comparing the CGH results between Ampli Taq LD and EHF DNA polymerase, the result with EHF DNA polymerase showed a remarkable improvement. The size and intensity of DNA fragments appeared larger (500 to 7000 bp), and stronger than those by Ampli Taq LD on the ethidium bromide-stained agarose gel (Fig 3.5.4-I). CGH profiles produced by product using EHF PCR system presented on nearly the 0 line without noise.

DNA from Known abnormal bone marrow samples

Table 3.5.11 Schematized strategy applied on DNA from abnormal bone marrow

Basic source of DNA	Amplification method	CGH result	Amount of template DNA	Enzyme used
Abnormal bone marrow with AML/ MDS	Hybridisation Method; amplified test DNA with non-amplified Nick-translated control DNA			
	PEP	fail	40 ng	AmpliTaq LD
		fail	4 ng	AmpliTaq LD
		fail	2 ng	AmpliTaq LD
	DOP-PCR	good	12 ng	Thermosequenase
		good	1.2 ng	Thermosequenase
		good	600 pg	Thermosequenase
		good	500 pg	Thermosequenase
		good	300 pg	Thermosequenase
		fail	100 pg	Thermosequenase
		fail	50 pg	Thermosequenase
Abnormal bone marrow (test DNA) Normal male blood	Hybridisation Method; amplified test DNA with amplified control DNA			
	PEP	Good	40 ng	EHF PCR system
	DOP-PCR	good	200 pg	EHF PCR system
		good	100 pg	EHF PCR system
		good	50 pg	EHF PCR system

This original DNA was extracted from abnormal bone marrow with AML/MDS, conventional karyotyping result was as follows:
46-47, XY, -5, ?der(9)del(9)(p13)inv9(p13q21.1), der(17)t(5;17)(p13;p11.2), +r, 8-13 dmin
The CGH result using original non-amplified DNA was loss of DNA copy number on the chromosome regions of 5q14-qter, 9p13-p22, 17p12-pter and amplification on 8q24.

1) Amplified test DNA hybridized with non-amplified, nick translated DNA

Amplification Method: PEP

PEP was performed with serially diluted known abnormal bone marrow samples.

As in the case of the normal female samples, two different sizes of primer (12 mer, 15mer) were used. The starting amount of template DNA was 40 ng, then 4 ng, and finally 2 ng.

The size of the amplified DNA fragments obtained from 40 ng of template DNA was from 500 bp to over 12000 bp (the latter mainly remained near the well). The amplified DNA fragments obtained from 4 ng and 2 ng of template DNA also remained near the well and were of very faint intensity on the agarose gel (Fig 3.5.4-K).

After labelling by nick translation, the size of DNA of all samples was over 500 bp with the major portion of DNA near the well.

These products amplified by PEP could not detect real abnormal copy number changes when used in CGH. The image usually showed less intensity of green (test DNA) than red (reference DNA) and over-represented heterochromatin-rich area.

Amplification Method: DOP-PCR

This experiment was designed to assess the reliability of whole genome amplification by DOP-PCR.

This sample had previously been analysed by CGH without amplification to allow comparison with results from with amplification. The original DNA concentration was 1μg/μl and the sample was diluted serially from 12 ng/50 μl to 50 pg/50 μl.

Table 3.5.12 showed abnormal findings from diluted bone marrow samples compared with known abnormal results.

The DNA smear from 12 ng of template DNA amplified by DOP-PCR showed fragment sizes from over 2000 bp to below 300 bp with strong intensity (Fig 3.5.4-L). The size of DNA amplified from 1.2 ng and 600 pg was also from over 2000 bp to below 500 bp with a strong intensity (Fig 3.5.4-F). After the first DOP-PCR amplification with 300 pg and 150 pg of template DNA, the size of DNA on agarose gel was 500 to 1600 bp with relatively strong intensity (Fig 3.5.4-G).

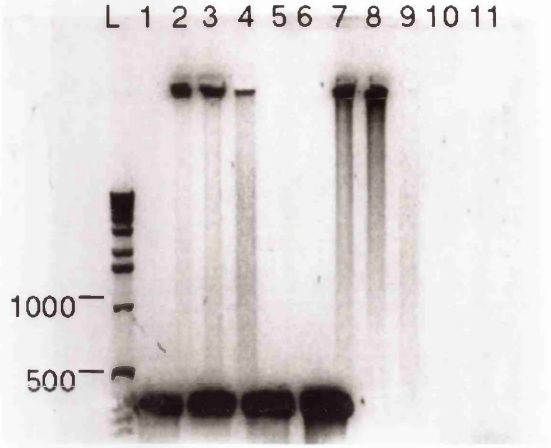


FIG 3.5.4 -K

Gel electrophoresis of the PEP products using AmpliTaq LD
PET; paraffin-embedded tissue
BM; bone marrow
L; 1 kb ladder
2; 4 ng of template DNA from abnormal BM (200 pmol)
3; 2 ng of template DNA from abnormal BM (200 pmol)
4; 4 ng of template DNA from abnormal BM (100 pmol)
7; 4 ng of template DNA from PET (200 pmol)
8; 2 ng of template DNA from PET (200 pmol)
9; 4 ng of template DNA from PET (100 pmol)
11; negative control

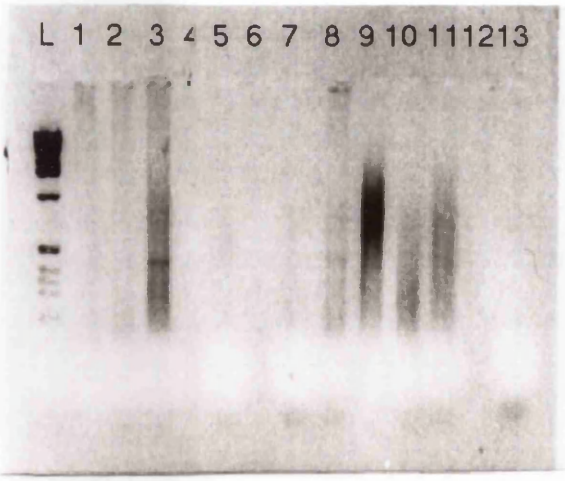


FIG 3.5.4 - L

Gel electrophoresis of the first DOP-PCR products using thermosequenase

L; 1 kb ladder
3; 12 ng of template DNA from abnormal BM
10; 12 ng of template DNA from PET
13; negative control

Above 500 pg of template DNA, this experiment showed relatively reliable CGH results but with under-represented regions on 1p33-pter, 19p, and 22. The intensity of amplification of 8q24 became weaker with decreasing amount of template DNA. But below the 300 pg level the reliability of CGH was markedly decreased. 1q33-qter, 19, 22, some telomeric regions appeared to be incorrectly over-represented but dark banding sites on G-banding seemed to be erroneously under-represented.

Actually under 100pg, this experiment could not produce any reliable abnormal finding. Below this level findings presented in below 300 pg level were more exaggerated and even amplification of 8q24 could not reliably identified.

Finally, at the end of this experiment, it was clear that an enzyme with proof-reading activity and optimization of amount of both test and control DNA for hybridisation were urgently required.

Table 3.5.12 Summary of CGH results from the serial dilution of DNA from abnormal bone marrow sample

Ch. No	gain	loss	12 ng	1.2 ng	600 pg	500 pg	300 pg	100 pg	50 pg
1			-1p33-pter	-1p33-pter	-1p 33-pter	-1p33-pter		+1p 32-pter	+1p 32-pter
2									
3									
4									
5		5q14-qter	±	±	±	+	±	?	?
6									
7									
8	8q24(A)		+>	+>	+>	+>	+	-	-
9		9p13-p22	-	-	-	-	-	+	+
10									
11									
12									
13									
14									
16									
17		17p12-pter	+	+	-17p	-17p	-17p	-	-
18									
19			-19p	-19p	-19p	-19p	-19p	+19	+19
20							+20p	+20	+20
21									
22			-22	-22?	-22?	-22		+22	+22

2) Amplified test DNA hybridized with amplified control DNA

Amplification method: PEP

In this experiment, amplified product using PEP with EHF PCR system from 40 ng of template DNAs (test and control DNA) only produced relatively reasonable CGH result, which showed distinct abnormal findings– loss of 5q, 9p13-p22 and amplification 8q24 except 17 p12-pter (Fig 3.5.4-M).

Amplification method: DOP-PCR

This study performed whole genome amplification with both test and control DNA samples using DOP-PCR, labelled during the second PCR in the same setting. EHF DNA polymerase was applied to whole DOP-PCR procedure. The starting amount of test DNA in the first DOP-PCR was 200 pg, 100 pg or 50 pg. The same amount of starting control DNA was used.

The DNA smear after the first DOP-PCR showed very large sized DNA (1000 to 5000 bp with maximum intensity) and strong smear (Fig 3.5.4-N). There was no significant difference when using different amounts of template DNA. By calculating DNA concentration of the product after second DOP-PCR, DNA concentrations also did not show a big difference between the three samples.

The CGH results from this study revealed more reliable, homogeneous finding with almost complete agreement with original abnormal findings (3 out of 4 abnormal findings) and did not show incorrectly over- or under- represented regions even on the 50pg level (Fig 3.5.4-O).

The summary of the result of this method comparing with original abnormalities is shown in Table 3.5.13.

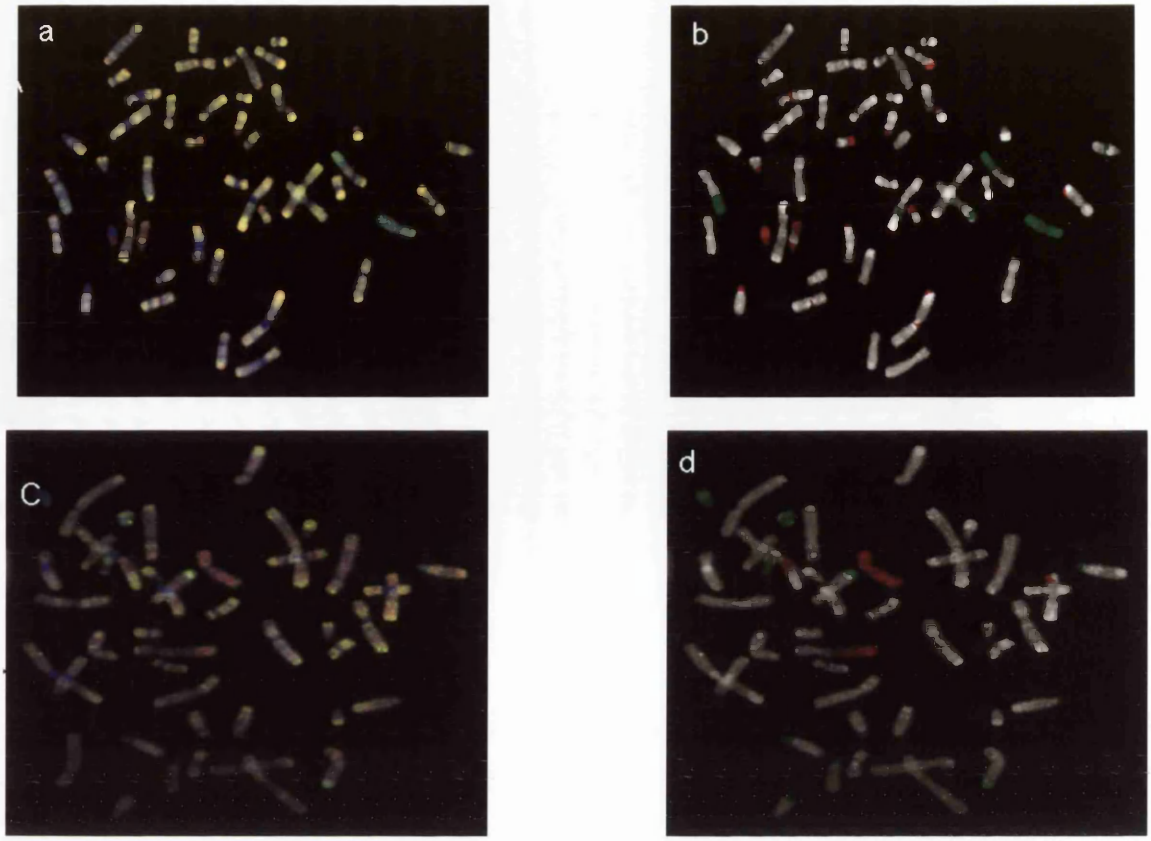


Fig 3.5.4-M (a)CGH image following colour equalisation (test DNA; 40 ng of template DNA from abnormal bone marrow sample amplified by PEP using EHF PCR system and labelled with Texas-Red, control DNA; 40 ng of template DNA from normal female blood amplified by PEP using EHF PCR system and labelled with FITC, and hybridised on normal male metaphases. (b) colour ratio image of the same metaphase (c)CGH image following colour equalisation (test DNA; 40 ng of template DNA from abnormal bone marrow sample amplified by PEP using EHF PCR system and labelled with FITC, control DNA; 40 ng of template DNA from normal female blood amplified by PEP using EHF PCR system and labelled with TexasRed, and hybridised on normal male metaphases. (d) colour ratio image of the same metaphase.

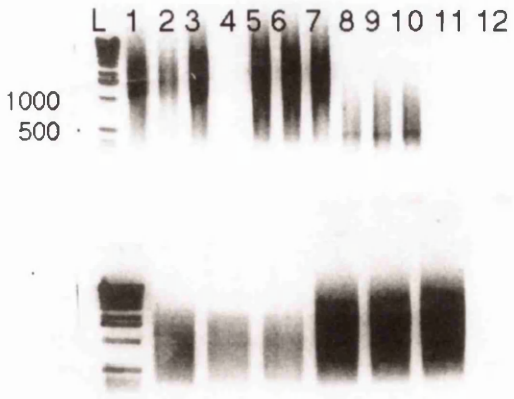


FIG 3.5.4 - N
Gel electrophoresis of the first DOP-PCR products with EHF PCR system
PET; paraffin-embedded tissue

L; 1 kb ladder
1; 200 pg of normal female DNA
3; 50 pg of normal female DNA
5; 200 pg of abnormal BM DNA
6; 100 pg of abnormal BM DNA
7; 50 pg of abnormal BM DNA
8; 50 pg of DNA from PET
9; 100 pg of DNA from PET
10;200 pg of DNA from PET
12; negative control

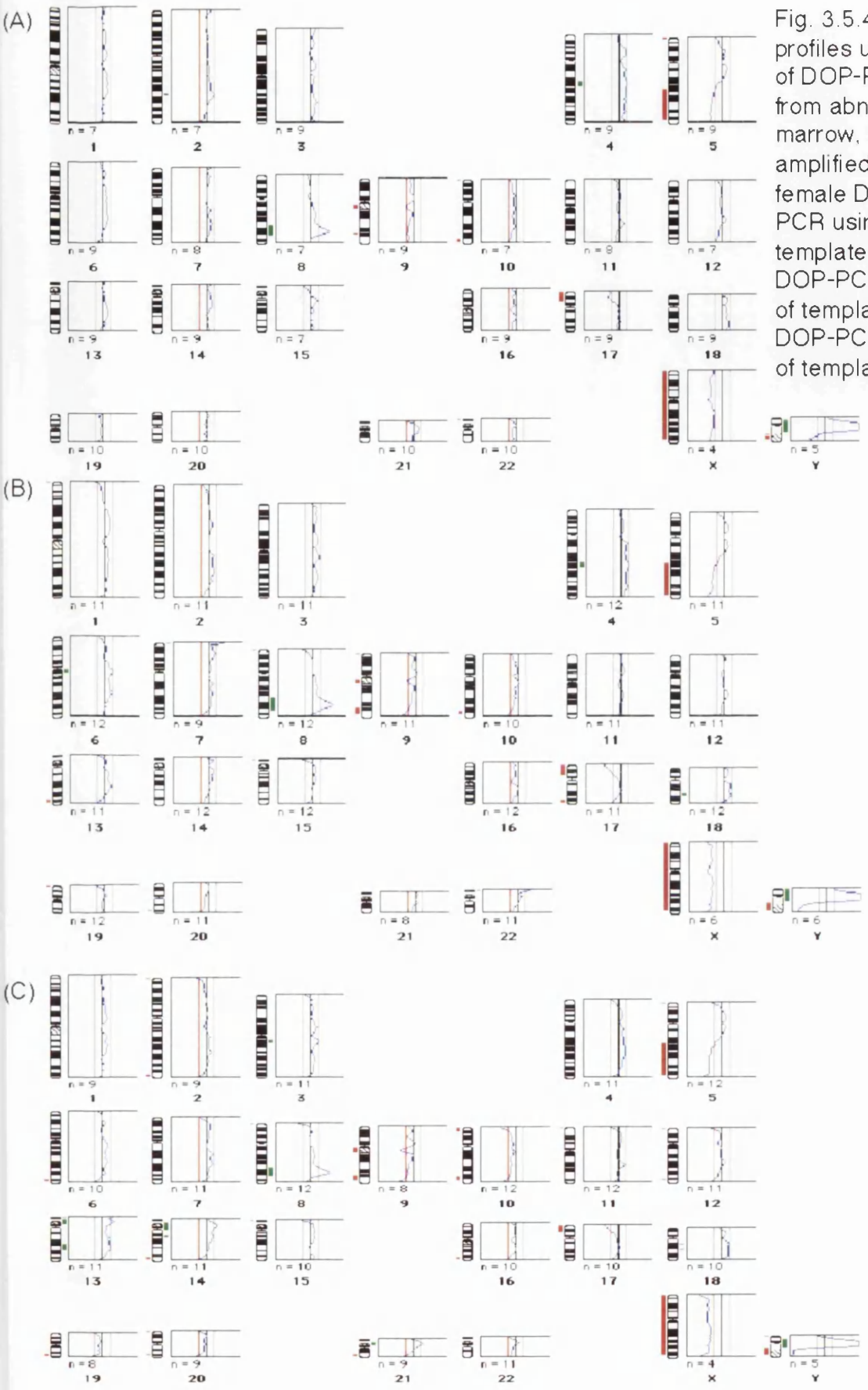


Fig. 3.5.4-O CGH ratio profiles using products of DOP-PCR with DNA from abnormal bone marrow, hybridised with amplified normal female DNA. (A) DOP-PCR using 200 pg of template DNA, (B) DOP-PCR using 100pg of template DNA, (C) DOP-PCR using 50 pg of template DNA

Table 3.5.13 Summary of CGH results from the serial dilution of DNA from abnormal bone marrow sample using the EHF PCR system

Ch. No.	gain	loss	200 pg	100 pg	50 pg
1					
2					
3					
4					
5		5q14-qter	-5q21-qter*	-5q21-qter*	-5q15-qter*
6					
7					
8	8q24 (A)		+ (decreased intensity)	+ (decreased intensity)	+ (decreased intensity)
9		9p13-pter	-	-	-
10					
11					
12					
13					
14					
15					
16					
17		17p12-pter	+	+	+
18					
19					
20					
21					
22					

-; cannot detect original abnormal finding

+; detect the same abnormal finding

*; Quite similar finding was detected but location of abnormal copy number changes was different

DNA from Known abnormal paraffin-embedded tissue samples

In this study, the basic DNA sample was extracted from paraffin-embedded ovarian cancer tissue and CGH was performed with non-amplified original DNA of this paraffin-embedded tissue.
The CGH result using original non-amplified DNA was losses of DNA copy number on chromosome 7p21-pter, 8p, 13q13-q21, 15q11.2-21.2, 16p12-pter, 17q, 18q21 and gains on chromosome 3q21-qter, 5p13-pter, 7q31-qter, 8q22-qter, 10q24-qter, 11q13-qter, and 12p.

Table 3.5.14 Schematized strategy applied on DNA from known abnormal paraffin-embedded tissue

Basic source of DNA	Amplification method	CGH Result	Amount of template DNA	Enzyme used
Paraffin-embedded ovarian cancer tissue (non-amplified)	Hybridisation Method; amplified test DNA with non-amplified Nick-translated control DNA			
	PEP	fail	40 ng	AmpliTaq LD
		fail	4 ng	AmpliTaq LD
		fail	2 ng	AmpliTaq LD
	DOP-PCR	good	12 ng	Thermosequenase
		good	1.2 ng	Thermosequenase
		good	1 ng	Thermosequenase
		good	600 pg	Thermosequenase
		good	500 pg	Thermosequenase
		fail	100 pg	Thermosequenase
Paraffin-embedded ovarian cancer tissue (test DNA) Normal male blood (control DNA)	Hybridisation Method; amplified test DNA with amplified control DNA			
	PEP	fail	40 ng	EHF PCR system
	DOP-PCR	good	200 pg	EHF PCR system
		good	100 pg	EHF PCR system
		good	50 pg	EHF PCR system

1) Amplified test DNA hybridized with non-amplified, nick translated DNA

Amplification Method: PEP

This study was performed in the same way as the normal female and abnormal

bone marrow samples. The original DNA sample was diluted to 40 ng, 4 ng, and 2 ng.

The size of amplified DNA fragments obtained from 40 ng of template DNA was from 300 bp to over 12000 bp. In the PEP amplification with 4 ng and 2 ng of template DNA, two different concentrations of primer (200 pmol and 100 pmol) were applied to the samples. The size of amplified DNA from 4 ng and 2 ng of template DNA with 200 pmol of primer concentration was spread from the well to 500 bp, but mainly remained near the well (Fig 3.5.4-K). After labelling by nick translation, these amplified products showed from high molecular weight DNA to below 300 bp and had a very faint intensity. In the case of amplification with 100 pmol of primer concentration, the size of the DNA fragments obtained from 4 ng of template DNA was from 500 bp to 12000 bp without high molecular weight DNA (Fig 3.5.4-K). After the labelling procedure this product showed below 500 bp sized DNA.

CGH with these amplified products could not reproduce the original abnormalities and showed a lot of non-specific background signals.

For reducing non-specific signals, CGH was performed with purified probes, but showed low intensity of green signals with slightly decreased non-specific background signals.

Amplification Method: DOP-PCR

The original DNA concentration was 1μg/μl. It was then serially diluted to 12ng, 1.2ng, 1ng, 600pg, 500pg, 100pg, 50pg of template DNA in 50μl of the first DOP-PCR buffer.

Above the level of 500 pg of template DNA, the first DOP-PCR products showed 200 to 1000 bp sized DNA fragments with a relatively strong smear. Below the level of 500 pg, DNA smear from paraffin-embedded tissue showed less intensity and smaller sized DNA than that of bone marrow on the same amount of starting DNA (Fig 3.5.4-G).

Above the level of 500 pg of template DNA, CGH results were also relatively compatible with original abnormal findings but with less intensity (detection rate; 70-85%) and there were some under- and over-represented regions. Disproportionately under-represented regions (-1p34-pter, -17p, -19, and -22) were more predominant than over-represented regions (+ 6q12-q16). From below that concentration, the detection rate of original abnormal findings was markedly

decreased (4 out of 14 abnormal finding) and under-represented findings presented in above 500 pg were started to change reversibly. The summary of the CGH results with serially diluted samples comparing with original abnormalities is presented in Table 3.5.15.

2) Amplified test DNA hybridized with amplified control DNA

Amplification method: PEP

In this experiment, PEP amplified product using EHF PCR system from 40 ng of template DNAs failed to obtain a proper CGH result.

Amplification method: DOP-PCR

DOP-PCR was performed with EHF DNA polymerase to improve the reliability. To avoid the problem induced by mismatched concentration of test and reference DNAs during hybridization, both samples were amplified by DOP-PCR, and labelled during DOP-PCR in the same setting. Although the size and intensity were weaker and smaller than other bone marrow or normal control samples, relatively good DNA (300-1000 bp) was revealed on an agarose gel running after the first DOP-PCR (Fig 3.5.4-N).
CGH on 200 pg and 100 pg of template DNA showed more reliable results in comparison with previous results. The CGH result with the amplified product obtained from 50 pg of template DNA showed about 64% of original abnormal findings with decreased sensitivity and accurate definition of abnormal regions (Fig 3.5.4-P).
The summary of CGH results in comparison with original abnormal findings is shown in Table 3.5.16.

Table 3.5.15 Summary of the CGH results from the serial dilution of DNA from paraffin-embedded ovarian cancer tissue

Ch.No	gain	loss	12ng	1.2ng	1ng	600pg	500pg	100pg
1			-1p34-pter	-1p34-pter	-1p34-pter	-1p34-pter	-1p34-pter	
2								
3	+3q21-qter		+	+	+	+?	+	-
4								
5	+5p13-pter		+	+5p*	+5p*	+5p*	+5p*	-
6			+6q12-q16	+6q12-q16	+6q12-q16	+6q12-q16	+6q12-q16	
7	+7q31-qter	-7p11.2-pter	+ -7p21-pter*	+ -7p21-pter*	+ ±	+ -7p21-pter*	+ -7p21-pter*	- ±
8	+8q22-qter	-8p	+ ±?	+ ±	+ +	+ ±	+ +	- ±
9								
10	+10q24-qter		+	-?	-	+?	-	-
11	+11q13.3-qter		+	+	+	+	+?	+
12	+12p		+	+	+	+?	+	+
13		-13q13-q21	-	-	-	-	-	+
14								
15		-15q11.2-21.2	-	-15q13-q15*	-15q13-q15*	-15q11.2-q15*	-15q13-q15*	-
16		-16p12-pter	+	+	+	-16p	+	-
17		-17q	-17*	-17*	-17*	-17*	-17*	-
18		-18q21	-	-	+	-	+	-
19			-19	-19p	-19p	-19p	-19?	-
20					+20p		+20	+20
21								
22			-22	-22	-22	-22	-22	

-; cannot detect original abnormal finding

+; detect the same abnormal finding

*; quite similar finding was detected but location of abnormal copy number change was different

± or ?; suspicious abnormal finding

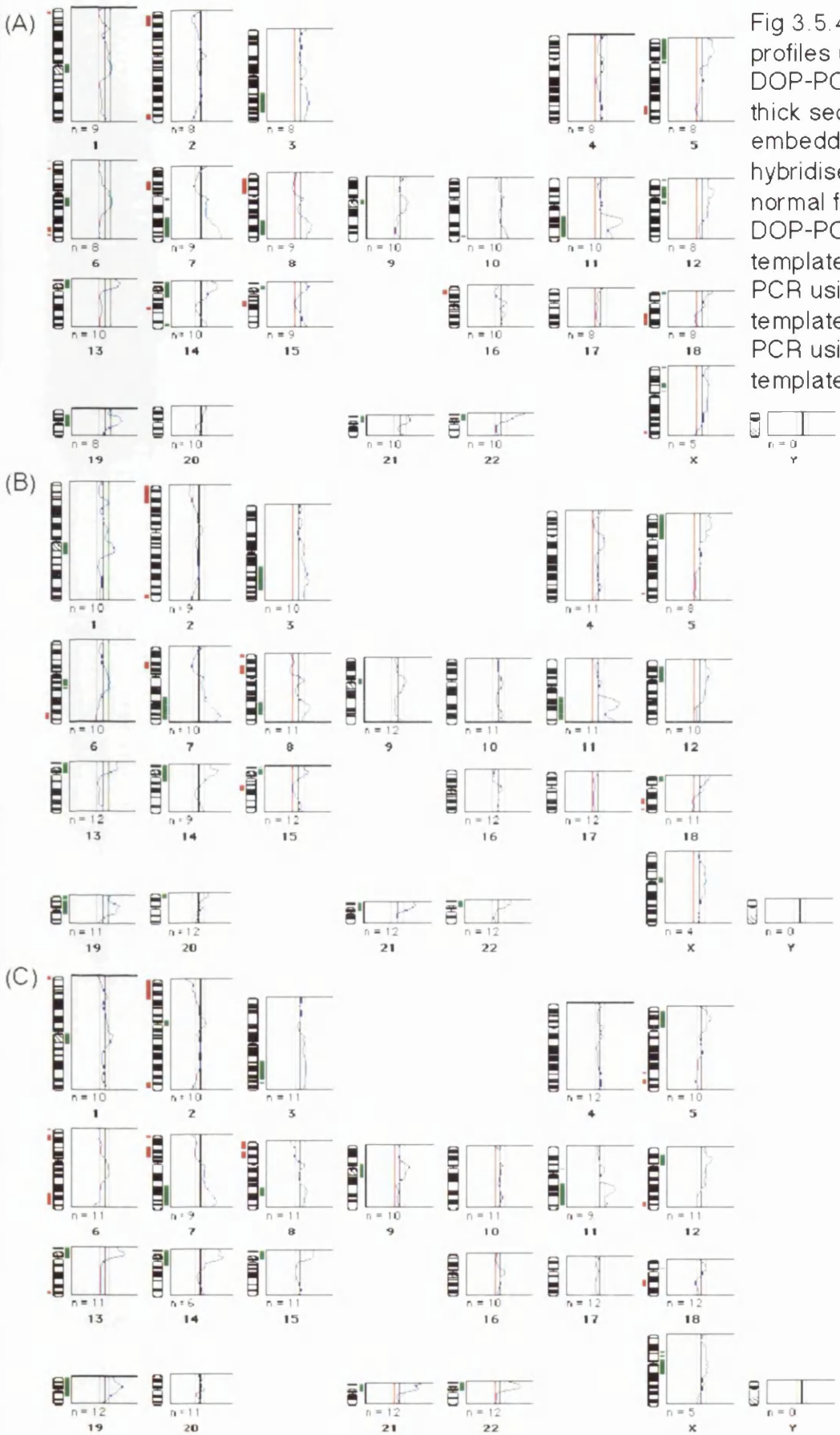


Fig 3.5.4-P CGH ratio profiles using products of DOP-PCR with DNA from thick section of paraffin-embedded tissue, hybridised with amplified normal female DNA. (A) DOP-PCR using 200 pg of template DNA, (B) DOP-PCR using 100 pg of template DNA, (C) DOP-PCR using 50 pg of template DNA

Table 3.5.16 Summary of the CGH results from the serial dilution of DNA from paraffin-embedded ovarian cancer tissue using EHF PCR system

Ch. No	gain	loss	200 pg	100 pg	50 pg
1					
2				-2p22-pter	-2p22-pter
3	+3q21-qter		+	+	±
4					
5	+5p13-pter		+	+	+5p14
6			+6q13-q15	+6q13-q15	
7	+7q31-qter	 -7p11.2-pter	+ -7p11.2-p14	+ -7p11.2-p14	+ -
8	+8q22-qter	 -8p	+ +	+ +	- -
9					
10	+10q24-qter		+?	-	-
11	+11q13.3-qter		+	+	+
12	+12p		+	+	+12p11.2-p12
13		-13q13-q21	-	-	-
14					
15		-15q11.2-21.2	-15q15-21	-15q15-21	-
16		-16p12-pter	+	-?	-
17		-17q	-?	-?	-
18		-18q21	+	+	-
19				+19p	+19p
20					
21					
22					

-; cannot detect original finding

+; detect the same abnormal finding

*; quite similar finding was detected but location of abnormal copy number change was slightly different.

?; suspicious abnormal finding

3.6 CGH experiments on very small amounts of DNA in solid tumours

3.6.1 Ovarian cancer

Section 3.5 has described the development of the technique using a very small amount of tissue from ovarian tumours. No cytogenetic analysis was possible from the tumour sections used, but the CGH results were consistent and showed abnormalities in regions known to be associated with ovarian cancer, in particular losses of chromosome regions 1p, 5, 11 and 15q, and gains of 1q and 12p. It therefore seems likely that these were true findings reflecting the chromosome constitution of the tumour tissue.

3.6.2 Malignant Melanoma

CGH with 1 x 7 μm whole tissue sections

The object of the various experimental trials just described was to obtain an analysable CGH image from the very small amount of DNA that can be extracted from paraffin embedded tissue. From this point onwards, the optimised method was applied to melanoma samples although some further improvements in the method were made during this study.

The numbering of these cases was as below:

Case 1: 972961 (male)
Case 2: 980804 (male)
Case 3: 981350 (male)
Case 4: 981746 (female)
Case 5: 982169 (male)

In the first experiment five paraffin-embedded melanoma samples were studied with DNA being extracted from one entire 7μm thick section from each. It is possible that the tumour DNA was contaminated with normal DNA from stromal cells.
At first, DNA from these samples was amplified by PEP. The DNA smear of

these samples after PEP showed different patterns for each individual sample. The size of DNA fragments in cases 1 and 2 was over 12000 bp, which mainly remained in the well (Fig 3.6.2-A). In cases 3, 4, and 5, the intensity of smear decreased sequentially from 4, 5, to 3. Although case 5 showed a reasonable size of smear as 500 bp-12000 bp without high molecular weight DNA in the well (Fig 3.6.2-B) the DNA smear appeared weaker and more fragmented after nick translation. Although CGH experiments after PEP were tried several times, it was not possible to obtain analysable CGH images except in case 5 which showed relatively analysable CGH images compared with those of the other cases (Fig 3.6.2-C).

The experiment was then repeated substituting the proofreading enzyme (Pfu DNA polymerase) for increasing the fidelity during PEP. The result of DNA amplification with this enzyme when visualized on ethidium bromide-stained agarose gels was similar to that with AmpliTaq LD. These amplified products also failed to achieve proper CGH images. In amplification using PEP, the main obstacle was to avoid contamination as already mentioned.

When DOP-PCR amplification was applied to DNA from the same five samples, cases 1, 2, and 3 showed very faint smears, especially case 3 which showed almost nothing on the agarose gel. In cases 1 and 2, the size of amplified DNA was from over 12000 bp to below 500 bp. Intensity of DNA smear of these amplified samples was weaker than the product from 600 pg of normal female DNA or 12ng of DNA from paraffin embedded tissue. Case 4 showed rather less but relatively similar intensity to the product from 600 pg of normal female DNA. The size of the amplified DNA isolated from case 5 was 2000 to 12000 bp with strong intensity (Fig 3.6.2-D).

In this experiment Pfu DNA polymerase was applied to whole genome amplification using DNA from these melanoma samples except case 5. Comparing the results between Pfu DNA polymerase and thermosequenase, cases 1 and 2 showed mainly below 500 bp sized DNA smear, case 3 showed still nothing on agarose gel, case 4 revealed 400 to 1000 bp with maximum intensity (Fig 3.6.2-E).

The first DOP-PCR products of these samples could not produce proper CGH results. The cause of failure could not be exactly evaluated because so many steps were involved, but the major cause of failure was the amount of template of DNA. After several trials, CGH produced analysable images. The summary of the results of these 5 cases is presented in Table 3.6.1.

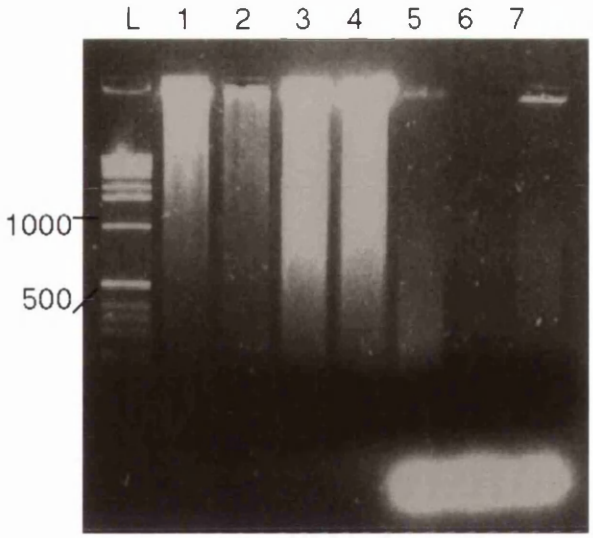


FIG 3.6.2-A

Gel electrophoresis of the PEP products with whole section of 1x7 um paraffin-embedded melanoma using AmpliTaq

L; 1 kb ladder
1; case 1
2; case 2
3; normal female control

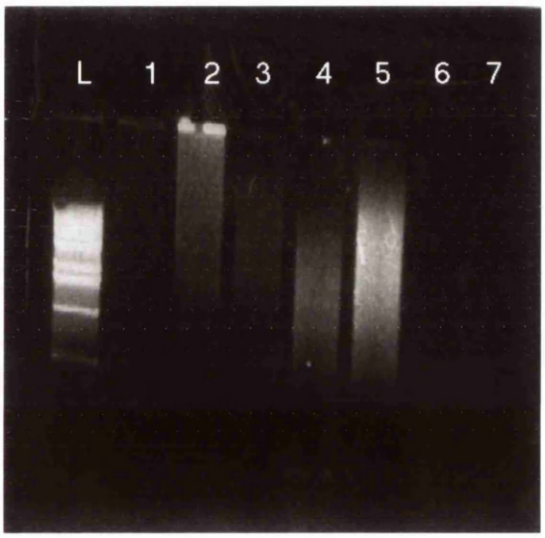


FIG 3.6.2-B

Gel electrophoresis of the PEP products with whole section of 1x7 um paraffin-embedded melanoma using AmpliTaq

L; 1 kb ladder
1; case 3
2; case 4
3; case 5
4; 40 ng of DNA from PEP
5; 40 ng of normal female DNA
7; negative control

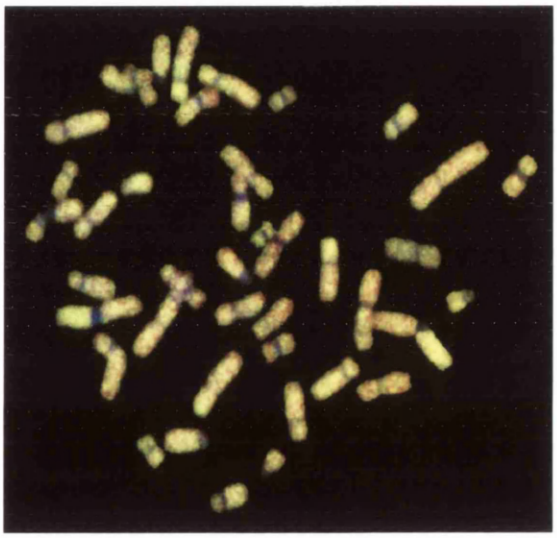


FIG 3.6.2-C CGH image following colour equalisation using case 5 PEP amplification product

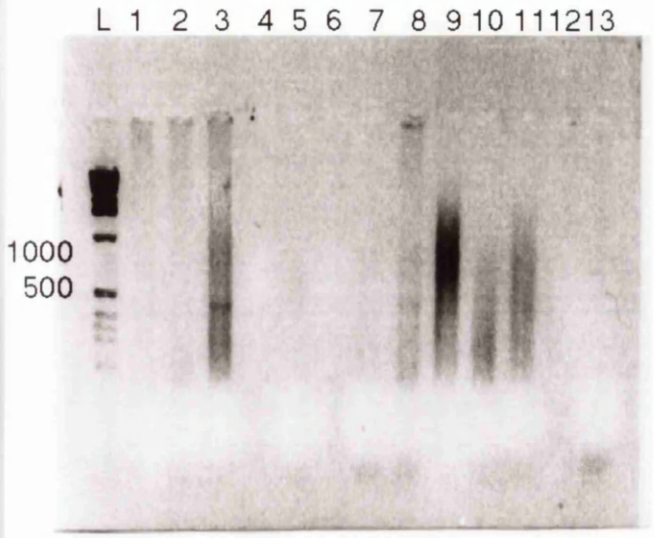


FIG 3.6.2 - D
Gel electrophoresis of the first DOP-PCR products with whole section 1x7um of paraffin-embedded melanoma tissue using thermosequenase
PET; paraffin-embedded tissue
BM; bone marrow

L; 1 kb ladder
1; case 1
2; case 2
3; 12 ng of abnormal BM DNA
5; negative control
7; case 3
8; case 4
9; case 5
10; 12 ng of DNA from PET
11; 600 pg of normal female DNA
13; negative control

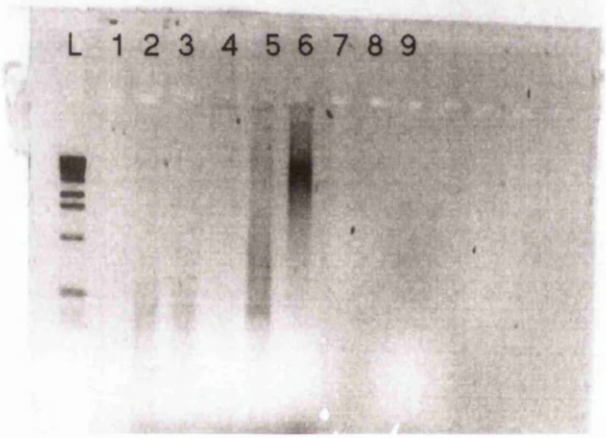


FIG 3.6.2 - E
Gel electrophoresis of the first DOP-PCR products with whole section 1x7um of paraffin-embedded melanoma tissue using Pfu DNA polymerase except case 5

L; 1 kb ladder
2; case 1
3 case 2
4; case 3
5; case 4
6; case 5
9; negative control

Table 3.6.1 Summary of the CGH results of five melanoma cases

(1x 7μm thickness of whole tissue sections)

Case number	gains	losses
1 (972961)	1q, 2p21-pter, 4p15.1-p15.3 7p12-p15.2, 7q31.32-qter, 9q21.2-q31, 12p12.2-p13.2, 15q22.3-q24, 17q23-q24	6q, 13q21-22.2
2(980804)	6p21.3-pter, 7q32.3-qter 15q22-q24, 16p12.2-pter 17q22-q24	9p, 13q21-q22.2
3(981350)	Failed	
4(981746)	Thermosequenase Pfu 6p24-pter 6p21.1-pter	Thermosequenase Pfu 6q12-q22 6q12-q22 9p21.1 9p21 13q14.2-31.1 13q14.2-31.1
5(982169)	1q, 4pter-q21	9p12-p22, 1p31.2-pter

All of these CGH results except case 5 showed many nonspecific findings such as over-represented regions on chromosomes distal 1p, 16, 19, 22, or some telomeric areas. Case 1 showed consistent results over several trial experiments (at least 5). Case 2 revealed matched sex chromosomes and true loss and gain such as loss of 9p, gain of 6p21.3-pter together with several suspected regions of gains and losses. However these suspected regions are difficult to present as true gains and losses because they occur in problematic regions when CGH is attempted on very small amount of template DNA. Case 3 failed to produce adequate CGH results and this was compatible with DNA smear finding. Case 4 showed some abnormal findings with the typical pattern which was shown in a very low concentration of starting DNA such as over-represented regions on chromosome 1p32-pter and 20 and under-represented G-dark band positive regions, especially chromosomes 4 and 5. Case 5 showed good quality of DNA on the gel and the CGH result was also very reliable and did not show unreasonably over-represented or under-represented regions.

Comparing the results between Pfu DNA polymerase and thermosequenase, case 4 only produced successful CGH results using Pfu DNA polymerase. Although the CGH findings with both enzymes were quite similar, the result using Pfu DNA polymerase revealed more reliable findings with matched sex chromosomes. Even though Pfu DNA polymerase was an on going optimization process, sensitivity of this enzyme was better than that of thermosequenase.

CGH with Microdissected Samples

Samples were microdissected from paraffin embedded melanoma tissue sections. Cells from the regions of interest were selectively microdissected from five paraffin-embedded melanoma tissue sections under the inverted microscope by using 25G needle. PEP products of these samples failed to produce successful CGH results even after many attempts, indicating that the size of the microdissected material was too small to produce a sufficient amount of amplified DNA for CGH. The WGA method was then changed to DOP-PCR.

At first, the size of microdissected area was 2 mm^2 x 3 μm which was composed almost entirely of abnormal cancer cells. Malignant cells were procured as much as possible without contamination of normal cells. The microdissected cells were treated with proteinase K to release the genomic DNA, which was amplified by DOP-PCR and directly labelled with FITC or Texas-Red. This amount of microdissected material failed to produce proper results.

After increasing the volume of microdissected material to 18 mm^2 x 3μm or 3 mm^2 x 3μm reasonable results were obtained. DOP-PCR method was changed from method 6 to method 7 due to repeated failure. The summary of the gel running results of amplification product (DOP-PCR) and the volume of the microdissected materials are shown as Table 3.6.2. The summary of CGH results of these 5 cases is presented in Table 3.6.3. All these experiments were repeated several times.

Table 3.6.2 Summary of the volume of the microdossected materials and DNA size after DOP-PCR amplification of microdissected samples from 5 melanoma cases

Case number	volume of the microdissected material	DOP-PCR product (1^{st})
1 (971354)	18 mm^2 x 3μm	Below 394 bp
2 (971496)	3 mm^2 x 3μm	Below 500 bp
3 (980804)	3 mm^2 x 3μm	300 to 1000 bp
4 (982186)	18 mm^2 x 3μm	300 to 1600 bp
5 (982963)	18 mm^2 x 3μm	300 to 1600 bp (300-500 bp with maximum intensity)

Table 3.6.3 Summary of the CGH results of microdissected samples from 5 melanoma cases (using AmpliTaq LD, hybridised with non-amplified control DNA)

Case number	Gains	Losses
1 (971354)	–	17p12-qter
2 (971496)	6p	10q22-q25.2
3 (980804)	6p22.2-pter, 15q22.3-q23 17q24	5q14-q32, 9p,10p,11pter-q13
4 (982186)	–	9p21-23
5 (982963)	5p, 6p, 22q11.2	6q, 9p21, 10p, 11q14-qter, 14q23-qter, 17p

*– no gain of DNA copy number

These CGH results showed abnormalities compatible with chromosomal aberrations which have commonly been observed in CGH of fresh melanoma samples. Gains most frequently involved chromosome 6p (3 out of 5 cases) and additional chromosomes with gains were chromosome 5p, 15q22.3-23, 17q24, and 22 (?). Losses most frequently involved chromosome 9p (3 out of 5 cases) and other chromosomes with losses were 10p, 17p13, and 6q.

Next, the method was modified by changing to an enzyme with proof-reading activity. Two systems were investigated- the Expand High Fidelity PCR system (Boehringer Mannheim) and the Expand Long Template PCR System (Boehringer Mannheim). The Expand High Fidelity PCR system is composed of a unique enzyme mix containing thermostable Taq DNA and Pwo DNA polymerase. The Expand Long Template PCR system, which is also composed of the same enzyme mixture as Expand High Fidelity PCR system, is designed to give a high yield of PCR products from genomic DNA. It amplifies fragments up to 27 kb from human genomic DNA.

At first, the PEP method was applied to case 5, with the Expand High Fidelity PCR system and the Expand Long Template PCR system, in comparison with normal female control (concentration; 4ng/50μl). The results of DNA amplification were visualized on ethidium bromide stained agarose gel (Fig 3.6.2-F). The size of amplified DNA with the EHF PCR system was over 12000 bp which mainly remained in the well, and showed less intensity than the product from normal female sample (concentration; 4ng/50μl). The size of the amplified DNA with the ELT PCR system was from 1000 bp to the well, but was uniformly spread. When CGH was attempted using the DNA amplified by PEP no reliable

results were obtained and this remained the same even after several attempts and modifications of thermal cycle and enzyme.

Then, the EHF PCR system and AmpliTaq LD were applied to DNA from the 5 cases during DOP-PCR amplification to compare the efficacy and fidelity. These amplified products were hybridised with amplified normal female control (amount of template DNA; 1 ng/50ul) using same enzyme.

At first, a $9mm^2$ x 3 μm thickness section was microdissected from paraffin-embedded tissue. This amount of microdissected material from each of the five melanoma cases was amplified by DOP-PCR using AmpliTaq LD and the EHF PCR system separately. The results showed that products of the EHF PCR system generally gave a stronger smear with a larger size of DNA fragments than those of AmpliTaq LD (Fig 3.6.2-G). Cases 1 and 2 showed very fragmented DNA (below 200 bp), The size of amplified DNA from cases 3 and 4 was 200 to 500 bp. Case 5 showed 300 to 1000 bp (around 500 bp with maximum intensity). Case 5 especially showed up a prominent difference between the results of the two enzymes (EHF; 300 to below 1000 bp, AmpliTaq; below 500 bp).

All cases except case 5 failed to produce acceptable CGH images, suggesting that they could not amplify sufficient DNA to produce an optimal CGH result. In case 5, the CGH result with the amplified product using AmpliTaq showed homogeneous, uniform images but was of lesser intensity than that of the EHF DNA polymerase. This method could only detect a gain of chromosome 5p and a loss of 14q23-qter when threshold was decreased to 1.12/0.88 but the results with EHF DNA polymerase were the most homogeneous and uniform yet produced (Fig 3.6.2-H).

After this the thickness of sections was increased to 7 μm to increase the yield of DNA amplified from microdissection of the same area (9 mm^2) except for case 2 (3 mm^2). It was thought that these amounts of tumour cells were amplified sufficiently for each CGH experiment. After DOP-PCR amplification, gel-running showed a slightly stronger smear and larger size of DNA fragments except in case 1 and 2 than those from previous experiment (Fig 3.6.2-I). All of these four cases except case 2 produced analysable CGH images. In case 2, the same volume as other samples could not be obtained because of the very small tumour mass on paraffin section and failed to produce a reasonable CGH result.

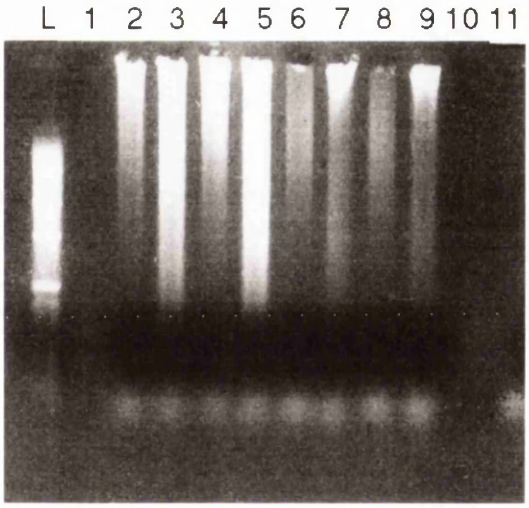


FIG 3.6.2 - F
Gel electrophoresis of the PEP products with microdissected melanoma sample using EHF PCR system (EHF) and Espand long Template system (EL)

L; 1 kb ladder
2; case 5 (982963) with EHF
3; 40 ng of normal female DNA with EHF
6; case 5 with EL
7; 40 ng of normal female DNA with EL
11; negative control

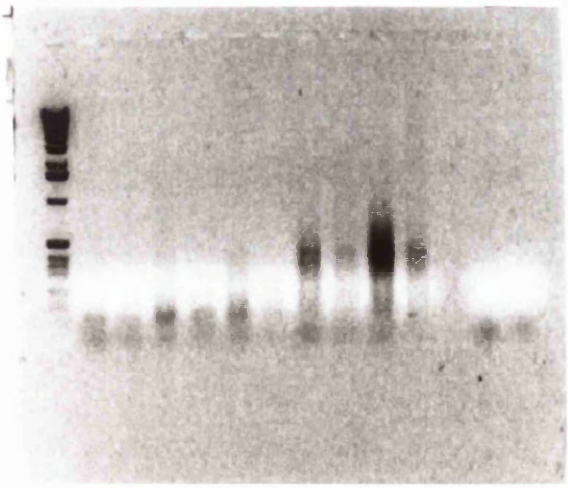


FIG 3.6.2 - G
Gel electrophoresis of the first DOP-PCR products with microdissected melanoma samples using EHF PCR system (EHF) and AmpliTaq LD (A)

L; 1 kb ladder
1; case 1 with EHF
2; case 1 with A
3; case 2 with EHF
4; case 2 with A
5; case 3 with EHF
6; case 3 with A
7; case 4 with EHF
8; case 4 with A
9; case 5 with EHF
10; case 5 with A
12; negative control
13; negative control

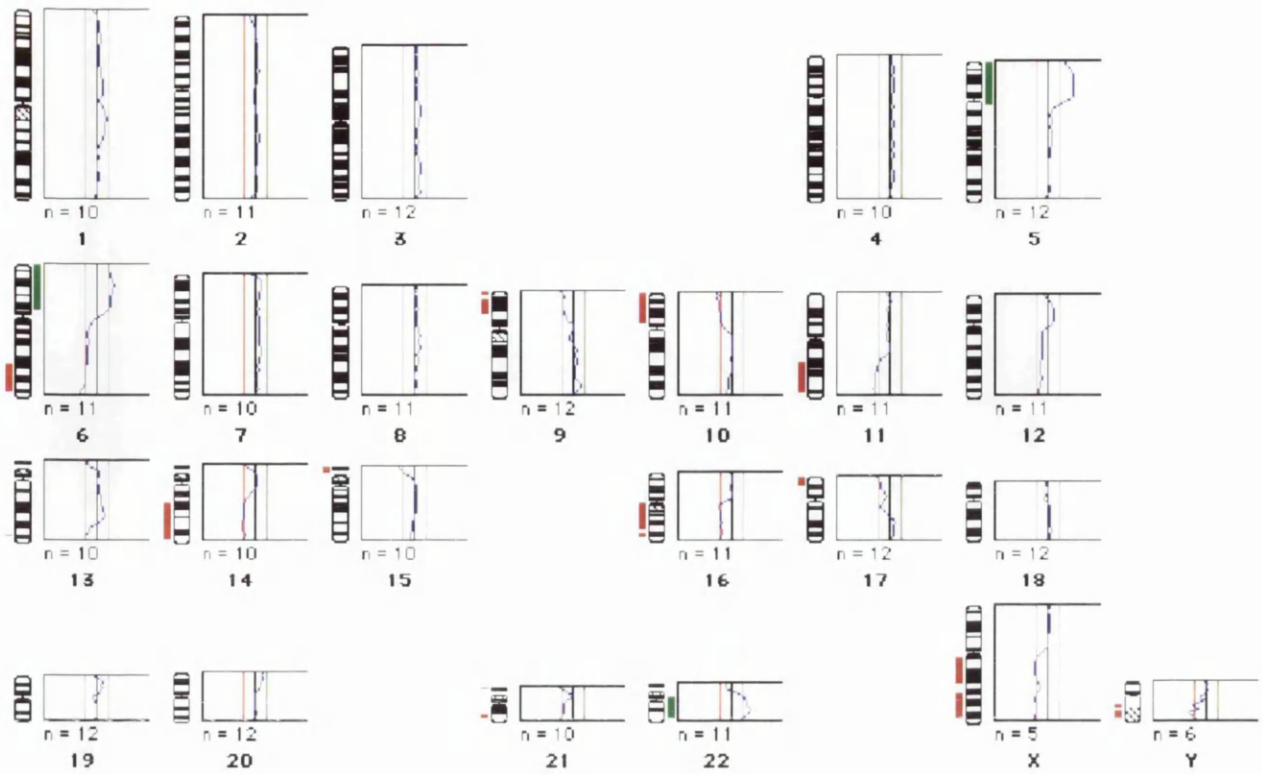


FIG 3.6.2-H CGH ratio profile using DOP-PCR product from case 5 (982963; 3mm x 3mm x 3 um)

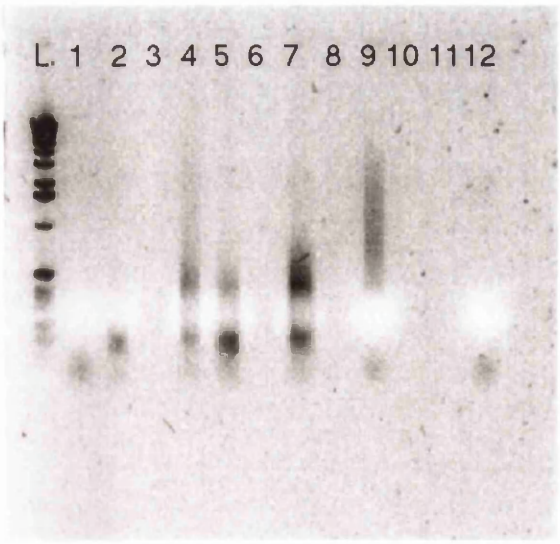


FIG 3.6.2 - I

Gel electrophoresis of the first DOP-PCR products with microdissected melanoma samples using EHF PCR system (3mm x 3mmx 7 um)

L; 1 kb ladder
1; case 1
2; case 2
4; case 3
5; case 4
7; case 5 (3mm x 3mm x 3 um)
9; normal female DNA (1 ng/50ul)
12; negative control

A summary of CGH results produced using these amplified products is presented in Table 3.6.4. All of these results were confirmed by repeating the experiments about five times and accompanied by various concentrations of normal and two positive controls to validate the results.

Table 3.6.4 Summary of the CGH results of microdissected samples of 5 melanoma cases (using EHF PCR system, hybridized with amplified control DNA)

Case number	Volume of microdissected material	gains	losses
1 (971354) (male)	9 mm^2 x 7μm		17p12-q22
2(971496) (female)	3 mm^2 x 7μm	Failed	
3 (980804) (male)	9 mm^2 x 7μm	6p21.3-p23, 15q22.3-q24, 17p12-qter	5q21.2-qter, 9p, 10p, 11pter -q22, 6q22-qter
4(982186) (male)	9 mm^2 x 7μm		9p21-pter
5(982963) (female)	9 mm^2 x 3μm	5p, 6p, 22	6q, 9p21-pter, 10p, 11q14-qter, 14q21.2-qter, 16q, 17p13, Xq

3.7 Validation of CGH results

The reliability of CGH results with amplified product, particularly from single cells or a few microdissected formalin-fixed and paraffin-embedded solid tumor cells, has not yet been evaluated. During whole genome amplification there are potential risks of false over- or under amplification of regions of DNA due to the intrinsic error rate of Taq polymerase. Such errors could produce some bias in subsequent CGH results.

Many methods have been applied to validate CGH results such as conventional karyotyping, FISH, and molecular methods. The fundamental way to obtain reliable CGH results is careful control of the quality of hybridization, and use of both normal DNA and DNA from known abnormal samples as controls. In this study, three control DNA samples from normal female, known abnormal bone

marrow sample, and known paraffin embedded tissue were applied as a control for the fidelity of the CGH results. All these control samples were amplified using PEP and DOP-PCR, labelled by PCR or nick translation, and CGH was performed along with the test samples of unknown composition. The results of all these control samples are mentioned in chapter 3.5.

3.7.1 Inverse (Reverse) CGH

Tumour samples were labelled with Texas-Red and hybridized with FITC-labelled normal reference DNA to control for the possible differential hybridization efficiency of differently labelled DNA probes.

Larramandy et al (1998) reported that chromosomal regions 1p33-pter, 16p, 17p, 19, and 22 exhibited hybridization artifacts in 15-23% of the tumour samples labelled with FITC, but not in samples labelled with Texas-red. However, in this study samples labelled with Texas-red showed similar pattern of over- and under-represented regions to samples labelled with FITC. Additionally, under-represented G-dark band areas were more exaggerated in reverse CGH.

Review of this inverse CGH showed many false positive and negative copy number changes when applying a modal value of 1.20/0.80. There were difficulties in differentiating whether these were true or false results. To increase the specificity, the threshold was widened to 1.40/0.60, and true copy number changes were decided by comparing with the original CGH images.

Case 5 had probably the highest concentration of DNA or the best condition (size of DNA). The original and reverse CGH results of this case were compatible with each other. They showed only a few over-represented chromosome regions namely the distal part of 1p, 19p, and 22.

Case 1 failed to produce a reverse CGH image.

Case 2, 3, 4 showed their original abnormalities with some discrepancies in addition to similar pattern of false positive and negative results, especially with more green (loss) on the dark G-band area and more red (gain) on chromosome 1p32-pter, 16p, 17, 19, 22. These findings suggested that the concentration of template DNA of case 2, 3, 4 was quite low or that optimal size of DNA fragments for CGH could not be produced sufficiently after amplification.

The results of reverse CGH and discrepancies between original CGH and reverse CGH are summarized in Table 3.7.1.

Table 3.7.1 Summary of the results of reverse CGH in five melanoma cases

Case No.	gains	losses	Discrepancies between two CGH results (original: reverse)
1	failed		
2	6p21.1-pter	9p13.2-pter	-9(p13.2-pter) (reverse) -10(q23.2-q25.2) (original)
3	6p21.1-pter, 15q22, 17q23-qter	9p, 10p11.3-p13	-5(q21.2-qter),-11(pter-q22), -16(q22-qter) (original)
4		9p13.2-pter	compatible
5	5p, 6p, 19p, 22	6q, 9p21-pter, 10p, 11q14-qter, 14q12 q24, 17p13, Xq	+19p (reverse), -16q (original)

3.7.2 Tissue In Situ Hybridization

The aim of this part of the study was to confirm the abnormal CGH findings in the five tumours studied. FISH was performed on 4 μm sections consecutive with those used for microdissection. Probes used were specific locus probe on *P53* region (17p13.1) with chromosome X centromeric probe as a control.

The main problem of this method is that in any given 4-um-thick section, portions of nuclei rather than intact nuclei are examined and exact counting is very difficult because of overlapping signals.

Case 1 (971354; male) consistently showed 1 signal for the control chromosome X centromeric probe. The result of *p53* locus specific probe showed 2 signals in the majority of nuclei but the presence of one signal and occasionally absence of signal was also detected. Cases 2, 3 and 4 showed similar results (Fig 3.7.2-A). In case 2, only *p53* locus specific probe was used. In this experiment, there are many difficulties to obtain proper results such as problems in counting the signals and availability of probes for specific locus interested.

3.7.3 PCR with specific primers

PCR using several different specific primers was also performed on the amplified products in an attempt to confirm these results.

PCR amplification using primer containing pseudoautosomal sequences was performed for sex determination of the samples and normal controls amplified

from 100 pg of template DNA was subjected to the same PCR amplification. This primer consists of a 30-mer derived from psudoautosomal sequences (C) in combination with 30-mer from Y-specific and X-specific regions (C:Y:X at a ratio of 50: 75: 5). Although specific bands were detected at 771 bp and 917 bp in male control and one band at 777 bp in female control, no specific bands were detected in the melanoma cases (Fig 3.7.3-A). Finally PCR using primer of *p16* gene on 9p21 (exon 2) was performed for proving the loss of *p16* gene. Although the strong single band was detected at 337 bp in normal control, no specific bands were detected in melanoma cases except case 3 (980804) which showed a faint single band at 337 bp, these findings were insufficient to reach a definite conclusion.

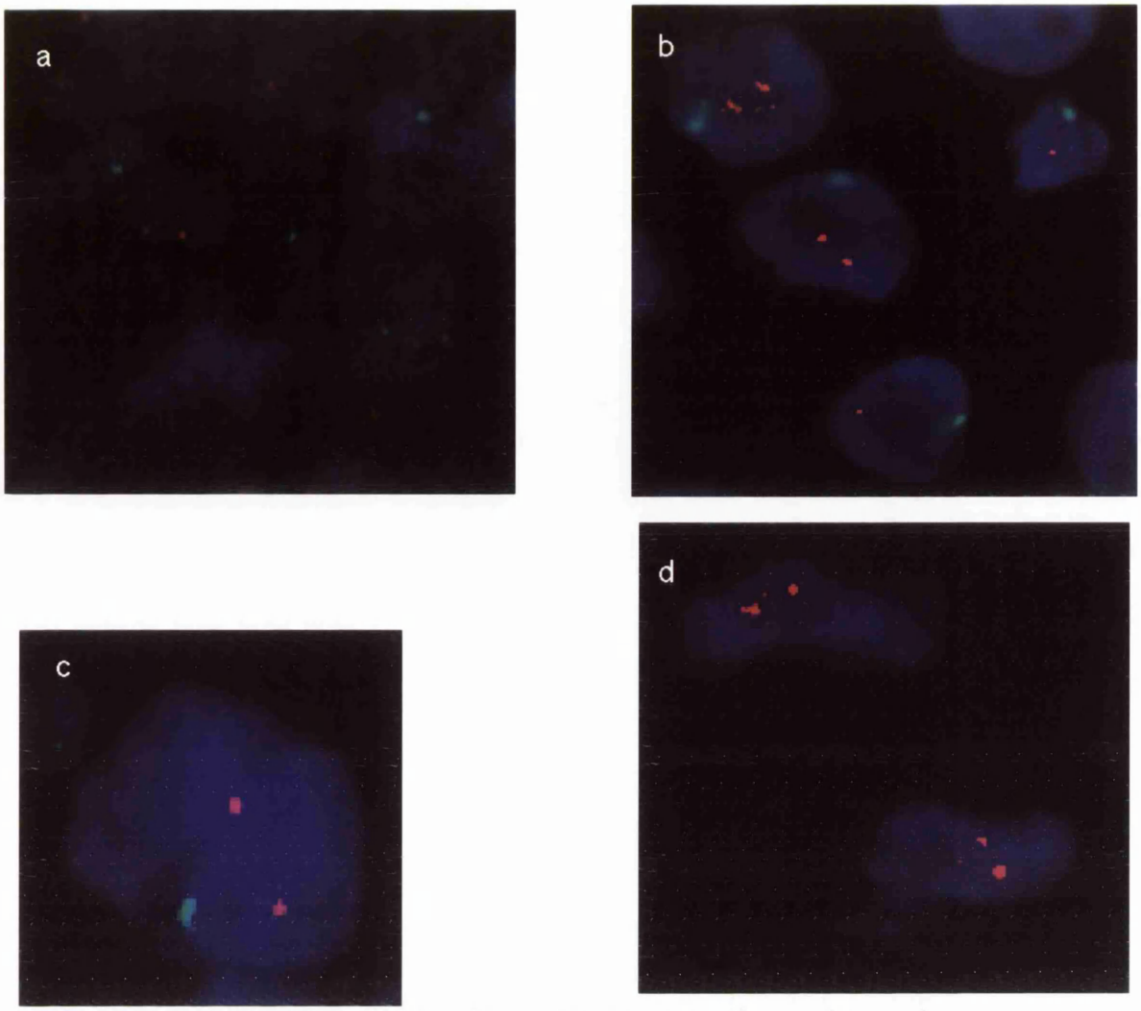


FIG 3.7.2-A Dual colour FISH using p53 locus specific probe (red)/ chomosome X centromeric probe (green) using paraffin-embedded tissue in three melanoma cases a) case 1 (971354), b) case 3 (980804), c) case 4 (982186) , Mono colour FISH using p53 locus specific probe (red) using paraffin-embedded tissue in one melanoma case d) case 2 (971496)

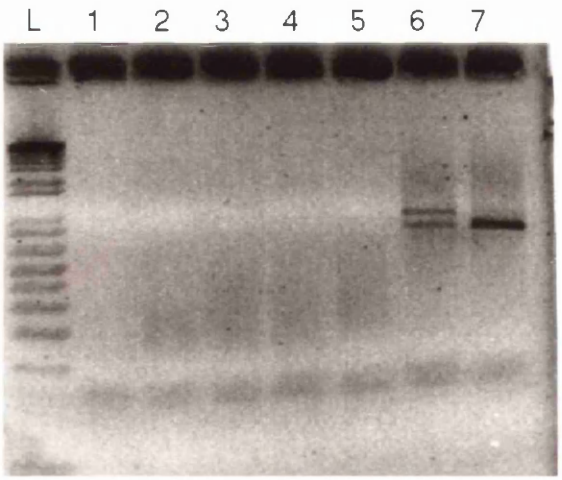


FIG 3.7.3-A

Gel electrophoresis of the PCR products using primer with specific sequences of pseudoautosomal regions of sex chromosomes in five melanoma samples and two amplified normal controls
L; 1 kb ladder
1; case 1
2; case 2
3; case 3
4; case 4
5; case 5
6; normal male (100 pg)
7; normal female (100 pg)

CHAPTER 4. DISCUSSION

4. Discussion

4.1 Interphase FISH and CGH in Acute Lymphoblastic Anemia

Fluorescent hybridisation using chromosome repetitive sequence probes is especially useful for the detection of numerical chromosomal abnormalities in prenatal diagnosis and is also of benefit in hematological malignancies especially leukemia because in many cases of leukemia it may be difficult to obtain any mitoses or good quality metaphase spreads.

The recognition of numerical and structural chromosomal aberrations has become an important part in the diagnosis of patients with acute lymphoblastic leukemia (ALL) because specific chromosomal aberrations in ALL affect therapeutic decisions and prognosis. The recent marked improvement in cure rates for children with ALL has induced a great interest about genetic abnormalities, including chromosomal number and nonrandom recurring chromosomal translocations as independent predictors of treatment outcome in childhood ALL. Nevertheless, cytogenetic analysis of ALL is difficult because standard chromosome-processing techniques frequently produce poor metaphases. In only 55-90% of cases with ALL can clonal chromosomal abnormalities be fully evaluated (Raimondi, 1993; Karhu et al., 1997b; Paszek-Vigier et al., 1997).

Although numerical changes in ALL occur in similar frequencies as in AML, they are difficult to detect systematically because of the variety of chromosomes involved in hyperdiploid ALL. The absolute number of chromosomes rather than the trisomy or monosomy of specific chromosomes correlates with prognosis. Therefore screening with only one or two chromosome specific probes is unlikely to result in clinically relevant information (Bentz et al., 1994b).

Hyperdiploid cases are divided into three ploidy subgroups according to their modal number; low hyperdiploidy (47-50 chromosomes), high hyperdiploidy

(51-68 chromosomes), and triploidy/tetraploidy (> 68 chromosomes). The good prognosis associated with high hyperdiploidy in children and adults is well established. The prognostic importance of low hyperdiploidy is less definite, and triploid/tetraploid cases appear to have a relatively poor prognosis. Chromosomal gain in hyperdiploidy has been shown to be nonrandom. Low hyperdiploid cases tend to gain chromosome X, 8, 10, and 21, whereas high hyperdiploid cases show nonrandom gain of chromosomes X, 4, 6, 10, 14, 17, 18, and 21 (Moorman et al., 1996).

Within these ploidy groups, the prognosis can be affected by specific chromosomal rearrangements, mainly the t(9;22) and the rearrangements of the MLL gene which confer a very poor prognosis whatever the ploidy group is, and the recently described t(12;21), which seems to have a very good prognosis (Shurtleff et al., 1995). Furthermore, not only the chromosomal number but also the nature of gained chromosomes are of prognostic value. Harris et al (1992) showed that the gain of chromosomes 4 and 10 was associated with a good prognosis, even in the low hyperdiploid subgroup.

Two approaches to the detection of hyperdiploidy were investigated in the present study.

In the first approach, samples from 22 patients were studied by interphase FISH according to the strategy of Moorman (1996). In this FISH employing chromosome-specific probes for alpha satellite, applied singly or together in the seven sets, could be used first to identify each hyperdiploid case and then to predict its ploidy subgroup.

In 22 cases studied here, interphase FISH detected 2 abnormal cases out of 5 cases which had failed by conventional karyotyping, and also resolved one hyperdiploidy case which had been analysed incompletely by conventional karyotyping.

In six cases where a normal karyotype had been found no abnormality was detected by FISH, thus adding confirmation to the cytogenetic result.

Of the four hyperdiploid cases which had been identified by karyotyping, three were confirmed by FISH, no further abnormalities being identified. In the fourth case, complete karyotypic analysis had not been possible but FISH

identified three of the chromosomes involved.

Although the number of cases studied here is small, it can be concluded that FISH, following Moorman's strategy, can contribute to the classification of cases of ALL according to ploidy, especially where karyotyping failed or is incomplete. Without selection of centromeric probes for the specific chromosomes known to be most frequently involved, this would be a slow and tedious process, although use of the centromeric multiprobe device developed after this stage of the work had been completed might simplify the analysis.

One of the major problems during interphase FISH for obtaining a reliable result was accurate counting of signals and avoiding the problem produced by overlapping of interphase domains. Additionally, it was very difficult to interpret mosaicism, so in every experiment the inclusion of a normal control was critical in deciding if the result was normal or abnormal.

Numerical abnormalities are sometimes accompanied by structural abnormalities and as the combination with structural aberrations such as t(9;22) or t(4;11) would affect the prognosis, a more comprehensive and straightforward way for detection of structural and numerical aberrations is needed. There is however no perfect method as even CGH has the limitation of failing to detect balanced translocations.

Interphase FISH can be used as a complementary method to detect prognostically important numerical abnormalities, especially hyperdiploidy, in ALL as a routine screening of cytogenetically failed cases. The strategy presented in this study provides a convenient method for selection of probes for this purpose.

Interphase FISH has another benefit in the evaluation of minimal residual disease in patients with ALL and a high hyperdiploid clone (>50 chromosomes) at diagnosis.

Some high hyperdiploid clones unusually shows an apparent ability to remain dormant for many years. Relapse in this subgroup, which occurs in approximately 25% of children and 35% of adults, tends to be after the completion of treatment and may occur even many years later (Kasprzyk and Secker-Walker, 1997). FISH of specific alpha satellite centromeric probes to

interphase nuclei provides the opportunity to detect small numbers of remnant cells that may be trisomic for these specific chromosomes. This presents the possibility of detecting how quickly the leukemic clone is eliminated in these patients after chemotherapy and therefore whether the rapidity of loss of the leukemic clone affects significantly in their long-term survival. Regular monitoring of the clone by FISH may enable early detection of a re-emerging clone. At relapse, interphase FISH may be able to estimate the size of the leukemic clone more accurately (Heerema et al., 1993; Bentz et al., 1994b; Kasprzyk and Secker-Walker, 1997).

The second approach was to investigate the application of CGH to the detection of hyperdiploidy. CGH represents a new, additional technique to evaluate chromosomal aberrations in pediatric ALLs, especially in cases where metaphases from the malignant cell clone are difficult to obtain and show apparently normal cytogenetic findings or complex karyotypes. The other major advantage of CGH is the ability to identify the origin of marker chromosomes and to resolve complex karyotypes (Karhu et al., 1997b).

Results were obtained for all the 19 cases studied by CGH. Chromosome gains were much more frequent than losses and there was some disagreement with conventional cytogenetic results. Cytogenetics revealed an abnormal clone in 6 cases out of 19 cases (excluding failed cases) whereas CGH detected further 2 abnormal cases. By observation of some discrepancies of results between the two methods, missing chromosomes in conventional karyotyping were thought to be rearranged into marker chromosome or derivative chromosome. On the other hand, some chromosomes which were lost or gained in conventional cytogenetics could not be detected by CGH possibly because of mosaicism with normal cells. Complete agreement of results between conventional karyotyping and CGH was found in 9 cases of the 19 cases studied using CGH.

Case 8 was of interest in that an additional Y chromosome was detected by CGH but this was not seen on conventional karyotyping. FISH then showed that the extra Y material was present in a marker chromosome. Additional Y material is very rare finding in ALL and its significance is unknown.

Case 13 showed an another feature of interest in the loss of DNA copy number of 6q11-q21 (interstitial deletion) and 9p13.2-pter identified by CGH compared with del(6)(q21), der(9)?t(9;21)(p13;q11.2) by conventional karyotyping. In ALL, deletion of 6q usually involves a proximal breakpoint between 6q13-q21 with distal breakpoints at q21 and q23, with common loss of band q21 (Barletta et al., 1987; Hayashi et al., 1990). Deletion of 6q is found as the sole abnormality in approximately 3% of cases but occurs more frequently with other abnormalities giving an overall incidence of 8% to 13% of childhood cases and 6% of adults (Secker-Walker, 1994). In this case breakpoints detected by CGH were different from those by conventional karyotyping. CGH can provide more accurate breakpoints than traditional cytogenetic method. Abnormalities of 9p have been reported in approximately 13% of ALL cases. Abnormalities of sequences from 9p21-p22 involve loss of sequences by deletion or unbalanced translocations. Two important established changes which result in 9p loss are i(9)(q10q10) and dic(9;12) (Secker-Walker, 1994). In this case (case 13), 9p was deleted together with translocation of the remaining chromosome to chromosome 21. Cytogenetically, this derivative chromosome was in query but it can be supposed that the derivative chromosome was probably dic(9;21) according to the CGH result.

In case 18, a very prominent peak at the 6p23-pter region was identified by CGH, suggesting a high-level amplification of DNA sequences originating from this chromosomal region. In ALL amplification is not a frequent finding and this region is not a frequently amplified region. Additional material of add(6)(p2?) by conventional cytogenetics was probably derived from material of 6p23-pter. But it was difficult to estimate how many copies were amplified in that region.

In case 20, conventional G-banding result showed very complicated results whereas CGH showed only one abnormality ie. gain of chromosome 8. By using FISH, chromosome 4 material was shown to be rearranged into add(3)(p25), der(11), add(17), and ring chromosome. This result proved that CGH can reveal exact true gain or loss, making it possible to resolve the origin of marker chromosomes or complex derivative chromosomes.

Among four cases, which were failed by conventional G-banding, one triploid case and one case with loss of Xpter-q24 were identified by CGH. One case of triploidy showed a limitation of CGH which cannot evaluate absolute copy numbers of chromosome regions. However if information from interphase FISH was combined full interpretation was possible.

Two cases of hyperdiploidy showed gains of chromosomes X, 4, 10, 14, 18, 21 commonly. Gains of these chromosomes were compatible with other hyperdiploidy. Although both cases have good prognostic factors in hyperdipoidy especially including chromosome 4 and 10, the prognosis in case 8 was difficult to predict due to the presence of marker chromosomes.

CGH has a great advantage of detecting unbalanced structural abnormalities in addition to hyperdiploidy in a single hybridization whereas FISH may require several experiments to detect hyperdiploidy and unbalanced structural abnormalities cannot be detected using interphase FISH. On the other hand FISH, when carefully controlled, can detect mosaicism more accurately, especially in lower percentages of tumour cells because mosaicism (< 50%) would be missed by CGH.

In the case with hypotriploidy/hyperdiplody, interphase FISH provided an important clue about the numerical abnormality because CGH can recognize only the difference of relative copy numbers, especially when karyotyping had failed to obtain proper results.

Finally, although this sample size was not large enough to confirm applicability and advantages of the two methods, both have different advantages and are complementary methods for each other. In this study, no attempt was made to identify specific balanced translocations such as t(9;22) or t(12;21) which are known to influence prognosis within hyperdiploid groups. While these could not be detected by CGH, it might be possible to use breakpoint specific probes such as *BCR/ABL* or *TEL/AML* in interphase FISH to improve the diagnosis.

Although FISH can be considered as a method to detect numerical abnormalities, specific balanced translocations, and to monitor residual disease, CGH can be a very comprehensive diagnostic tool for detecting hyperdiploidy, unbalanced abnormalities, and amplification in ALL. Furthermore, prognosis

was estimated more accurately by detecting the exact breakpoint and rearranged chromosomal material as an origin of marker chromosome or derivative chromosome using CGH.

4.2 CGH analysis in cases with AML/MDS

Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS)-related AMLs are a very heterogeneous group of hematological diseases which show a diverse spectrum of chromosomal aberrations, molecular abnormalities and immunophenotypes.

In patients with acute myeloid leukemia, about 20%-40% of patients never achieve remission following standard induction chemotherapy. Only 30-40% of patients who achieve remission will eventually become long term survivors in spite of treatment with intensive chemotherapy, bone marrow transplantation or both (de Nully Brown et al., 1997a; El-Rifai et al., 1997). Therefore it is beneficial to allocate patients to different risk groups to prevent disease relapse, and to increase long term survival rates. There are many parameters which may influence the prognosis including age, FAB type, clinical variables especially presentation white cell count and the expression of certain immunophenotypic surface markers, but appropriate analysis of chromosomal abnormalities is one of the most valuable prognostic indicators in AML. Chromosomal abnormalities have been reported in between 50 and 95% of patients with primary AML in different studies (Bentz et al., 1995a; de Nully Brown et al., 1997; Musilova et al., 1998). AML patients with specific balanced re-arrangements such as t(15;17), t(8;21) or inv(16) have a relatively good prognosis. AML patients with complex karyotypes, often including several marker chromosomes, especially those with monosomy 5 or del(5q), monosomy 7 or abnormalities of 3q have been regarded as a poor prognostic group (Swansbury et al., 1994; de Nully Brown et al., 1997; Hirons et al., 1997; Grimwade et al., 1998; Musilova et al., 1998; Burnett et al., 1999).

The results of CGH on the 19 cases studied showed that net gains and losses of chromosome material could be detected more simply and accurately by CGH than by conventional karyotyping in patients with a complex karyotype. This study has shown that chromosomes thought to be missing by conventional karyotyping can be internally rearranged within the karyotype by incorporation into marker chromosomes or derivative chromosomes which have been partially identified.

Loss of chromosome 5q material is the most frequent finding in these CGH results (6 out of 19 cases). Three of these 6 cases presented with de novo AML. Five cases had loss of 5q as part of a complex karyotype. The remaining case had failed by cytogenetic analysis. Loss of 5q material has been reported to be particularly frequent in therapy-related or secondary AML/MDS and is less common in de novo AML (< 10%) (Horrigan et al., 1996). Although the size of deletion of 5q varies among the patients studied, a region that includes band 5q31 was always deleted except for case 17. Loss of 5q31 within the hematopoietic stem cell compartment could lead to initiation and clonal evolution of MDS/AML because it could be crucial to the preservation of genomic integrity following DNA damage (Beretta et al., 1996a; Horrigan et al., 1996; Wang et al., 1997; Karp, 1998).

In 4 cases of unbalanced t(5;17) leading to loss of material from chromosomes 5 and 17, CGH defined the break points relative to the centromere of each chromosome more accurately than cytogenetics had done. Three of the cases had been reported as having a der(5)t(5;17) or der(17)t(5;17). One of these three cases appeared to be dicentric by CGH.

When amplification of a specific locus has been identified by CGH, FISH with an appropriate probe can detect its distribution within the karyotype. Although the incidence of DMs in AML is only 1-5%, this finding is more frequently observed in de novo AML and MDS of the elderly, therapy-related or secondary AML/MDS and in a subset of a newly defined FAB M2/M4 leukemia characterized by trisomy 4, associated with clonal abnormalities of chromosome 5 and 7 (Slovak et al., 1994; Grimwade et al., 1998). In this study one case (case 4) is a secondary AML M4, associated with t(5;17), monosomy

5q , deleted 9p and 17p and the presence of double minutes (amplification of *MYC*). In case 7 seven copies of the *MLL* gene locus were confirmed by FISH. The amplification of an oncogene in secondary leukemia often happens in advanced stages of the disease and is regarded as a poor prognostic sign (Schwab et al., 1990; Mohamed et al., 1993; Slovak et al., 1994; Zhang et al., 1995).

In case 12, where conventional karyotyping reported a del(7)(p13p15) or add(7)(p13) and loss of chromosome 21, CGH identified loss of chromosome 7p12-pter without loss of chromosome 21. This was confirmed as a dic(7p;21p) by FISH. It is known that monosomy 7q is a critical marker of secondary leukemia, but the prognostic and clinical implication of monosomy 7p, dic(7p;21p) cannot be evaluated at the moment. This case also showed most cells to have similar related abnormalities in particular showing further re-arrangement of chromosome 5, 8 and 17. In this case, despite evidence of clonal evolution, CGH followed by FISH was able to detect underlying true gains and losses.

In this study, results with targeted FISH following CGH analysis confirm that the origin of marker chromosomes may be complex with two or more chromosomes involved in their make up. For example in case 12, one of the marker chromosomes was composed of material from chromosome 3, 18, and 22. Analysis of different metaphases may help to identify the evolution of the marker chromosomes from a simple stem line during the course of disease progression.

In case 13, CGH followed by FISH showed one marker chromosome to be composed of material from chromosome 6, 8 and Y. Conventional karyotyping by contrast had indicated only loss of the Y chromosome. In the literature review, several cases of loss of chromosome Y in association with a complex karyotype in AML patients have been reported (United Kingdom Cancer Cytogenetic Group, 1992). Further careful CGH studies in these cases is required because it may reveal instances where the Y chromosome is involved in the make up of the marker chromosomes which has hitherto not been reported.

Although the clinical importance and the events leading to the production of marker chromosomes is not yet fully understood, these experiments show that they are connected with complex unbalanced karyotypes and are usually associated with a poor prognosis.

The present study confirms the importance of CGH in the detection of some critical prognostic indicators in patients with AML or MDS transformation. It is especially useful in cases with extremely complex chromosomal aberrations and in cases which had failed by conventional karyotyping. Identification of the chromosome regions involved may also help to clarify their role in the pathogenesis of the disease.

4.3 CGH analysis in case with Well Differentiated Liposarcoma

Liposarcoma is one of the most common soft tissue sarcoma types in adults. Well-differentiated liposarcoma is one of the major categories of liposarcoma, and is characterized by supernumerary ring and giant rod-like marker chromosomes as a cytogenetic finding. Knuutila et al (1998) identified 12q14-q21 (7/30), 1q21-q24 (5/22), 1p33-pter(1/14), 19(1/14), Xp21(1/14) as amplification sites in liposarcoma but the frequencies of amplification were not subdivided by liposacoma subtype.

In this study, 5 cases of WDLPS showed ring chromosomes or rod-like giant marker chromosomes on G-banding. In four out of the 5 cases, amplification on chromosome between 12q13 and 12q21 of variable extent was observed. By comparison with conventional karyotyping, it was thought that this amplified material was incorporated into the ring chromosomes or giant marker chromosomes with or without other amplified material.

This region (12q13-21) is a quite specific amplification site in different kinds of sarcoma such as liposarcoma and osteosarcoma. This amplicon is very complex with the presence of several different genes such as *MDM2*, *SAS*, *CHOP/GADD153*. *MDM2* and *SAS* are constantly amplified in WDLS and

CHOP/GADD153 is rearranged in myxoid liposarcoma (Szymanska et al., 1996). Although the common amplification site involved is in 12q13-15, sometimes amplification in 12q14-15 and 12q21.3-22 are detected as two separate bands or as a broad amplified segment ranging from 12q14 to q22 (Suijkerbuijk et al., 1994; Wolf et al., 1997)

In cases with gain of material on chromosome 1, Szymanska et al (1996) found that common regions of gain were located at 1p22-31, 1q21-24, and 1q32 (with similar results from Suijkerbuijk et al., 1994).

In this study, case 3 showed two peaks of gain at 1q21-24 and 1q31 represented as an amplification band (1q21-24) and a high level of gain (1q31) simultaneously which was a relatively rare finding compared with amplification on each region separately.

In case 1, conventional G-banding showed hypotriploidy with a derivative chromosome and a marker chromosome. Initial CGH (before multiple normalisation of single chromosome) showed gain of chromosomes 8, 18, and 11p14.3-pter, loss of chromosomes 2, 4, 9, 11, 14, 15, X, and 5p15.3-pter and amplification of 12p13-21. By considering both results, the origin of add(5)(p14).ish der(5)(wcp12+) and mar.ish der(?)(wcp12+) could be proposed. This case also revealed the documented limitation of CGH analysis about absolute copy numbers.

The most interesting finding of this study was amplification of chromosome 3q12-13.3, which is thought to be a new amplification in liposarcoma. This amplification was detected in 2 serial samples of the same patient with 4 months time difference. In the cytogenetic view, one ring chromosome was added to previous findings but the CGH result was not changed. This finding suggested that two amplicons were constantly activated for progression of the tumour.

These results indicate that CGH is very helpful for evaluation of marker chromosome and ring chromosomes by the detection of amplicons and can therefore provide a clue about candidate oncogenes or tumour suppressor genes.

4.4 CGH analysis in cases with malignant melanoma

Although previous cytogenetic, molecular genetic, and linkage studies have discovered some genetic changes involved in the pathogenesis of melanoma, knowledge about genetic changes in sporadic cases is still limited (Bastian et al, 1998). It is very important to know about genetic changes from primary lesion to metastatic lesion because it may allow evaluation of the risk of metastasis in a primary melanoma and to differentiate potential precursors in benign lesions.

In this study, DNAs of 17 cases were extracted from fresh frozen tissue, which had some possibility of contamination with normal stromal cells. This could induce false aberrations to be detected by CGH.

Commonly involved chromosomes such as 10, 6, 9, 8, 7, 17 were relatively in agreement with the results of previous cytogenetic studies of melanoma (Ozisik et al.; 1994, Thompson et al.; 1995, Bastian et al.; 1998). Ozisik et al (1994), in a cytogenetic study of 21 malignant melanoma samples showed very complicated banding results with many marker chromosomes and/or derivative chromosomes. In comparison with previous cytogenetic or CGH analysis, the results in their study showed some discrepancies, which were caused by internal rearrangement of some chromosomal material into marker chromosome or derivative chromosomes.

Although chromosome 1 is highlighted to contain tumour suppressor genes, represented by deletions (breakpoints between p32 and p36) or translocations (breakpoints in p22 and p36), this study showed randomly scattered gains over the entire length of the chromosome without any loss of chromosomal material. The fact that 1p11.2-p12 region and 1q21 showed simultaneously high degree of gains of copy numbers (amplification on 1p11.2-p12) even with the suppression of the centromeric regions with unlabelled Cot-1 DNA was thought to be significant, even though this region is near the centromere.

Chromosome 3 was known to be a less frequently involved site in cutaneous melanoma rather than uveal melanoma. In case 6, a high level of gain on 3p24-p25.2 including amplification on 3q24 was noted. In another sample from this

tumour taken after 7 months and 14 days, this high level of gain could not be identified and was replaced by other new abnormalities such as gain of 7, loss of material of 2p, expanded lost region of 9, and loss of 13q22-qter. This finding suggests that the involvement of chromosome 3 probably appears earlier than involvement of chromosome 7, 2, and 13 although just one case is not sufficient to reach any conclusion. In other cytogenetic studies, involvement of chromosome 3 with breakpoints in p11-p25 is usually found in primary cutaneous melanoma.

Chromosome 6 was one of the most frequent involved chromosomes in this study. Short arm of chromosome 6 involvement was identified in 7 out of 17 cases, the most frequent overlapping region was 6p23-pter. Gain of 6p was almost always accompanied with loss of 6q. Degree of involvement was relatively similar in both arms of chromosome 6. The copy number change in chromosome 6 was accompanied with loss of chromosome 9p in nearly all cases. The frequent gain of the p arm indicates that a possible oncogene involved in melanoma progression resides on the distal part of the p arm of chromosome 6. The q arm of chromosome 6 has been shown to harbour a suppressor gene related to the pathogenesis of melanoma (Bastian et al., 1998).

Chromosome 7 was also frequently involved, always as gain of DNA copy number from the whole length of chromosome 7 to near the telomeric regions. One case showed amplification in 7q31.2-qter, which has been reported before (Wiltshire et al., 1995). One of the interesting findings was that chromosome 7 involvement was frequently associated with loss of 13q (minimal overlapping region of 13q22). It has not yet been determined whether this relationship was meaningful or just a random finding. As mentioned before, one case which had been studied again after more than 6 months showed gain of 7 as a new chromosomal change, indicating that it is a later change in tumour progression (Wiltshire et al., 1995; Bastian et al., 1998). Bastian et al (1998) reported that chromosome 7 was probably involved in later stage of tumour because they found statistical significance of chromosome 7 involvement in thicker tumour.

Although gain of chromosome 8q is one of the most common chromosomal aberrations in uveal melanoma, in our study, 6 out of 17 cases showed gain of

8q and 2 out of 17 showed loss of 8p. Loss of 8p was always accompanied with gain of 8q. Bastian et al have suggested that a tumour suppressor gene relevant in melanoma may be sited in the 8p21-pter region, and one case of this study also showed involvement of same site. Gains on chromosome 8 always involved the long arm, with a minimal overlapping region of 8q24-qter, where the *MYC* oncogene is located.

Involvement of chromosome 9p was one of the most frequent finding in this study, which highlights it as a location of a candidate gene of the early stage of melanoma and this was especially marked in cases with familial melanoma. In this study, the common deleted area of chromosome 9 encompassed 9p13-p22 including 9p21 which contains the *p16* gene. Involvement of chromosome 9 in this study was not the sole abnormality, which was frequently accompanied with many other aberrations including loss of 10 and involvement of chromosome 6. From the literature review, some papers documented that loss of 9p was associated with loss of material in chromosome 10, and suggested that both findings were early changes in malignant melanoma (Indsto et al., 1997; Bastian et al., 1998). However, it is impossible to estimate the time sequence of chromosome involvement in this study because of the lack of clinical information about the samples. Even if loss of 9p is an earlier finding in malignant melanoma, this study showed loss of 9p was probably sustained in later stages too.

In chromosome 10 involvement, the most frequently lost area was the q arm. In cases with p arm involvement, the distal part of 10p14 was frequently involved. According to Indsto et al (1997), all deletions of 10 showed codeletion of 9p in their study. This was not a constant finding in the present study. Chromosome 10 is also suspected to harbour a tumour suppressor gene for progression of malignant melanoma because loss of 10q is also important in pathogenesis of gliomas.

Chromosome 11 was shown to have random involvement. One case with a high degree of gain of 11q12.3-q13.1 was identified. This region is an amplification site in breast cancer and contains several candidate genes such as *CYCLIN D1/PRA D1, BCL1* (Hesketh, 1995; Knuutila et al., 1998).

In this study, chromosome 17 was frequently involved, especially the distal part of 17q with one amplification at 17q24-qter or several high level of gains at 17q22-qter. Amplification on 17q24-qter has been identified in different organ systems such as testicular cancer, MPNST (malignant peripheral nerve sheath tumour), and in lung cancer and amplification on 17q25 in melanoma (Bastian et al., 1998; Knuutila et al., 1998). It is suggested that the distal part of chromosome 17 (17q24-qter) possesses many important genes involved in the pathogenesis of melanoma or other tumours and this region may be another possible region to search for new candidate oncogenes.

Loss of chromosome Y was noted in 3 cases of this study. It is interesting that this finding is a phenomenon caused either by normal aging process or by tumour progression.

Finally DNA copy number changes were observed in chromosome 16, 19, 20, 22. Although these regions show widely variable fluorescence ratio in CGH, gain of chromosome 22 was interesting because one case showed very strong amplification of this chromosome. In previous melanoma studies, chromosome 16 and 22 have also been suggested as chromosomal changes in the early stages (rapid growth phase) (Thompson et al., 1995).

Although the overall copy number changes identified in this study were in agreement with other previous studies, several interesting findings were observed and highlight four amplified regions, namely chromosome 1p11.2-p12, 3p24, 17q24-qter and all of chromosome 22, that might harbour unknown genes involved in melanoma pathogenesis.

Involvement of 10 q and 17q where putative tumour suppressor genes (10q) and putative oncogenes (17q21-22) are contained is very interesting because both regions are also involved in glioma and embryologically melanocytes are of neural crest origin. These findings suggest that pathogenesis of melanoma may be closely related with other neural crest-derived tumours. In this study 3 cases showed simultaneous involvement of both regions (high level of gain of 17q with minimal overlapping area on 17q22-qter and loss of 10q with minimal overlapping area on 10q22.2-24).

4.5 Various kinds of DNA extraction methods from paraffin-embedded tissue

Various kinds of DNA extraction methods were applied in this study by changing several parameters. Actually, the success rate for obtaining high quality and quantity of DNA depends both on status of the sample and on parameters applied during the extraction process. As DNA in paraffin-embedded tissue was already fragmented and extensively cross-linked with nuclear protein, there are several important steps in the process of DNA extraction.

First the de-paraffinization step was performed in two different ways, depending on whether or not preincubation was included before the dewaxing step. Extending the preincubation step beyond three hours may have no beneficial effect on samples according to this study because prolonged incubation produced drying of the samples. Although DNA extraction after three hours incubation did produce high molecular weight DNA, there was no significant difference of CGH results between the DNA extracted with and without preincubation.

Secondly, it appears that proteinase K digestion may prove to be the most important step during DNA extraction from paraffin-embedded tissue with respect to degrading the nuclear protein involved in chromosome packaging. Modifications in the initial concentration, the length of incubation time and the addition of further proteinase K during incubation were introduced depending on the size of the sample. Six different incubation times (4 days, 3 days, 2 days, 24 hours, 3 hours, 90 min) were investigated. With a large sample, 3-4 days incubation with an initial concentration of proteinase K of 0.3 mg/ml and addition of further enzyme after 24 hrs and again after 48 hrs was found to give good results. In the case of a small sample, best results were found with an incubation period of 24 hrs or less and an initial concentration of 1-2 mg/ml. The results also indicate that when a short incubation is to be used (3hrs or 90 min), it may be beneficial to increase the temperature from 55°C to 65°C.

Third, some components of the buffer solution such as SDS and EDTA can affect the efficacy of PCR. Although SDS is an ionic detergent well known for its protein denaturing effect, it was found that even at a concentration of 0.005% SDS decreased the yield of PCR product dramatically. In the presence of ≥0.5 mM EDTA, the yield of PCR product was reduced. In this experiment, although other factors also affected the yield of PCR product, experiments including these components failed to produce adequate PCR product. Finally it was found that when a phenol/chloroform step was included, DNA extracted by this method consistently failed to produce sufficient DNA after amplification and successful CGH, while the same method but omitting this step, gave a high rate of successful CGH. Phenol, used for the denaturation of proteins such as protease and nuclease, and for the removal of solutes, is known to decrease the yield of PCR product when present at a final concentration over 0.2%. The cause of failure in this study was thus thought to be that the phenol was not completely removed. Another cause of exclusion of phenol/chloroform extraction is that the size of the sample was too small to perform the two step extraction method. Although crude DNA without phenol/chloroform extraction also could obtain successful PCR results, presumably if some amount of protein still remained in the crude DNA, then this remnant protein also might disturb PCR amplification. In future studies, it would be better to try a final extraction with chloroform or chloroform/isoamyl alcohol to remove residual phenol if sample is large enough.

Speicher et al (1993) suggested that an additional incubation step with sodium thiocyanate, a strong protein-denaturating agent which dissociates DNA-nucleoprotein complexes, introduced after the dewaxing would significantly help to increase the DNA yield and efficiency of subsequent DOP-PCR. However, some papers reported that they had similar successful results without pretreatment with sodium thiocyanate (Isola et al., 1994; James and Vally, 1996).

Although many different DNA isolation methods were investigated in this part of the thesis, it appeared that the most critical factor in obtaining an adequate amount of DNA was the size of the initial sample to be extracted.

4.6 Labelling the amplified probe

Although it is important to match the average length of DNA fragments in both test and reference samples, this may be difficult if the DNA from the formalin-fixed samples is somewhat degraded to begin with (James, 1999).

During the labelling procedure of the amplified products, two serious problems were noted in this study. First, the labelling efficiency either by nick translation or by PCR labelling was very difficult to estimate. This problem was more serious when amplified products were hybridized with non-amplified, nick translated reference DNA because the percentage of fluorochrome incorporated into the DNA during both labelling procedures was unknown in addition to the difference in quality between the test and reference DNA.

Following from this, the second problem was the difficulty in deciding how much reference DNA should be hybridised with a given amount of test DNA since an unbalanced amount of optimally sized DNA fragments between the two DNAs might produce a biased CGH result. For production of a successful CGH result, not only the same amount but also the same quality of both DNAs was required. Although minor differences can be overcome by the normalisation process during image processing, beyond a certain degree of tolerance, biased results and cross-talk phenomena may be produced.

To solve these problems, the percentage of fluorochrome incorporated into the probes was roughly calculated by using purification methods and semi-quantitative labelling assays as described in the Results section. The optimal amount of test DNA/control DNA also needed to be considered carefully due to different probe penetrating abilities. After calculation of labelled DNA concentrations, the ratio of test/control DNA was increased sequentially from 1:1, 1.5:1, 2:1, 2.5:1, to 3:1. When 2-2.5 fold of test DNA was hybridised with control DNA, an acceptable CGH result was obtained.

Thereafter, the problems caused by differences between efficiency of both labelling methods were solved by using hybridisation with simultaneously amplified control DNAs. By using this method, CGH image became more

homogeneous, uniform and reliable but still needed adjustment of the ratio of test/control DNA.

In many papers describing CGH after WGA of DNA obtained from paraffin-embedded sections, no mention is made of the relative amounts of test and reference DNA employed or whether differences in probe penetration were encountered. However, in the present study, it proved impossible to achieve good CGH images by using equal amounts of test and reference DNA because probe made from paraffin-embedded tissue did not have as same amount of suitable sized DNA fragments as normal control DNA and also the amount of fluorochrome incorporated into the DNA could not be calculated accurately.

In the future, this problem can be solved by using microdissected normal cells from the same section as a control DNA. For achieving this, more precise method for obtaining pure normal cell and tumour cell mass such as laser microdissection and close interdepartmental relationship with pathology are absolutely required.

4.7 Universal amplification of DNA

4.7.1 Degenerate Oligonucleotide Primed PCR (DOP-PCR)

Whole genomic amplification is a valuable technique to produce an increase of DNA from limiting amount of template DNA in a sequence dependent fashion. Various kinds of whole genome amplification have been developed including PEP, Alu-PCR, linker adaptor PCR, tagged-PCR, and DOP-PCR.

IRS-PCR has notable advantages for species-specific amplification. In human DNA, the major family of short interspersed repetitive sequence group (SINES) is the Alu family, which is believed to number 900,000 elements in the haploid genome, giving an average distance between copies of 3-4 kb. Although these repeats are found ubiquitously in human DNA, Alu sequences appear to be enriched in certain chromosomal regions and deficient in others. Alu elements

are preferentially found in the G-light bands of human chromosomes (Korenberg and Rykowski, 1988), therefore hybridization using chromosome paints amplified by Alu-PCR showed as a banding pattern (Telenius et al., 1992).

Linker adaptor PCR consists of several steps including digestion with a frequent cutter, ligation to an oligonucleotide, and amplification of the ligated product with primers specific to the oligonucleotides. Because there should be very little sequence selection bias with linker-adaptor PCR, except on the basis of distance between restriction sites, the technique overcomes the problems of regional bias and species dependence seen in IRS-PCR (Cheung and Nelson, 1996).

Tagged-PCR uses primers with a random sequence at the 3′ end to allow binding to a wide variety of target sequences and with a constant 5′ tail. The first round of amplification is similar to primer extension preamplification to allow many priming sites. After several rounds of non-specific interactions, unbound primers are removed, and PCR amplification proceeds with a primer specific to the 5′ constant region to allow exponential amplification (Cheung and Kelson, 1996).

DOP-PCR was developed as a simple PCR technique involving multiple locus priming, which allows a more general amplification than IRS-PCR, to allow an unselected amplification of any source DNA, and results in a more uniform CGH signal than interspersed repeat-based methods of whole genome amplification.

The efficiency of amplification of DNA by DOP-PCR depends on two fundamental requirements; initial low annealing temperature cycles which allow the primer to initiate PCR from short target sequences, and primer degeneracy. The six degenerate positions create a pool of 4^6 primers of different sequences, against the single sequence of a nondegenerate primer.

While the DNA of frozen tissue sections can be extracted as high molecular weight DNA for DOP-PCR with direct, standard cell lysis protocols, it is very difficult to obtain an adequate amount of good quality of DNA from paraffin-

embedded tissue due to interactions of formalin between DNA and proteins during the formalin fixation. When molecular or cytogenetic studies using DNA from paraffin-embedded tissue are being performed, artifacts due to the formalin interactions or the DOP-PCR itself should be considered (Speicher et al., 1993).

In this study DOP-PCR was first carried out by Guan's method (Guan et al., 1993) with topoisomerase 1 and T7 sequenase, which was used for microdissected chromosomal materials. Although the use of topoisomerase followed by sequenase in the low stringency cycle steps gave better results than any modification with the Taq DNA polymerase in previous reported studies, there was no significant benefit related to topoisomerase 1 pretreatment in this experiment. That could possibly be explained by the less condensed nature of DNA in interphase nuclei which more easily allows access of enzymes to the template DNA.

Then, to improve the size of amplified DNA and to decrease the chance of contamination, the thermostable form of sequenase, thermosequenase was used in this study (Kuukajärvi et al, 1997). This method was thought to be more sensitive than the topoisomerase + T7 sequenase combination on the basis of the final CGH result using this method (method 6) which gave images which were almost analysable although the template DNA had been obtained from only one section of 7 μm thickness. Another important benefit of using thermosequenase is that there is no need for repeat pipetting because thermosequenase is extremely thermostable. The use of standard sequenase was complicated because new enzyme has to be added after the denaturation step of each amplification cycle due to its thermolabile nature. Repeated pipetting gave an increased chance of contamination (Kuukajärvi et al, 1997).

After moving to experiments with very small amounts of DNA, proper amplification using method 6 sometimes could not be accomplished. To increase the efficacy of DOP-PCR with very small amounts of DNA, thermal cycling conditions were readjusted by increasing the number of low annealing temperature cycles. AmpliTaq LD was used as an enzyme to avoid the chance of contamination because the first PCR solution for DOP-PCR using Amplitaq

LD remained during whole DOP-PCR amplification process. The problems in this method were the characteristics of AmpliTaq LD such as less thermostability and a high error frequency because of lack of 3′-5′ exonuclease activity. Theoretically, when this amplified product is to be used for CGH, the result is probably not seriously affected because polymerase-induced mutations are randomly distributed over the amplified fragment and CGH can identify DNA copy number changes over 3-5 Mb (Cha and Thilly, 1995).

Finally an enzyme which combines proofreading activity with thermostability was needed to increase the reliability of CGH. Pfu DNA polymerase and Expand High Fidelity PCR System (Boehringer Mannheim) were investigated for this purpose. Although Pfu DNA polymerase has more fidelity than EHF PCR System, EHF PCR System, which is a mixture of thermostable Taq DNA polymerase and Pwo DNA polymerase, was finally used as enzyme because it was more easily optimised.

Compared with AmpliTaq LD, the results using EHF PCR System with the same sample and thermal cycle conditions showed stronger and longer DNA smear in agarose gel, and increased sensitivity and specificity in CGH results. This suggests that a proofreading enzyme is desirable for amplifying very small amounts of DNA or poor quality DNA to increase the reproducibility and reliability.

Methods 1 and 5 used the same DOP-PCR method; the only difference was in the amount of template DNA (method 1; 1x7 μm, method 6; 3x7 μm). Method 1 failed to amplify properly, method 6 showed relatively ideal size of DNA and more homogeneously hybridized CGH images, which revealed definite gain of chromosome 12p, some suspected abnormal findings and matched sex chromosomes. This finding suggested that the amount of template DNA profoundly affected the success of DOP-PCR amplification.

In this part of the study, normal reference DNA was not amplified, and was hybridized after labelling by nick translation. Therefore, the erroneously over- and under- represented regions in the amplified test product could be recognized when compared with non-amplified normal control, but CGH

profiles were very noisy and granular.

CGH results from methods 1 and 2, using DNA amplified by DOP-PCR, showed quite similar patterns. Although green signal (test DNA) showed less intensity than red signal (normal control DNA), chromosome regions 1p32-pter, 19, 16p, and 22 were over-represented and G-dark band compact areas were relatively under-represented under proper suppression. Probably, there are some reasons why those regions are preferentially amplified. These over-represented regions are all relatively pale areas by G-banding. It has been shown that Alu repeats are preferentially located in such regions which are known to be GC rich. Conversely, G-dark band areas are rich in AT and contain relatively fewer Alu repeats (Holmquist et al., 1982; Korenburg and Rykowski, 1988). These results suggest that when the template DNA was at an extremely low concentration, Alu or GC-rich sequences might be preferentially amplified. False over-representation was also observed at the telomeric regions of some chromosomes including 7, 8, 9, and 10 in contrast to the finding in standard CGH analysis that telomeric regions exhibit decreased fluorescence intensity. This finding also suggests that pale band regions in the end of the chromosome were amplified preferentially. Also this regional variability has to be considered with non-uniform denaturation, hybridisation properties of the different regions of the metaphase, and the quality of the two labelled DNAs such as labelling efficiency and size distribution of the fragments.

Even though the combination of DOP-PCR and CGH has many limitations, this method will allow the analysis of genomic imbalances in tumours with only a few cells or even eventually a intact single cell microdissected from paraffin-embedded tissue as starting material in association with the other techniques such as laser microdissection to select a pure population of cancer cells. It also makes possible a comparison of genetic changes with histological and immunochemical features and evaluations of the multiclonality of a tumour and evaluation of progression of genetic changes from early stage to metastatic tumour.

4.7.2 Primer-extension preamplification (PEP)

PEP is an another method of WGA, which involves multiple rounds of extension with Taq DNA polymerase and a random mixture of 15 base oligonucleotides as primers. It produces multiple copies of the DNA sequences originally present in the sample. It is estimated that PEP cover at least 78% of the genomic sequences in a single human haploid cell and can amplify any sequence in the genome to a minimum 30 copies (Zhang et al., 1992).

In this study, experiments were performed to assess the capability of PEP amplified DNA to produce CGH images. The variables investigated were amount of template DNA, temperature and number of thermal cycles, cell lysis method, and enzyme used.

First, it appeared that the most critical factor in PEP was the amount of template DNA. In these experiments, one 7 μm thickness sections of paraffin-embedded ovarian cancer tissue were used as a source of test material. Successful CGH images were obtained several times from these samples, but not consistently. However when melanoma samples of the same thickness were used as test material without microdissection, only one case produced an adequate amplified product to produce proper CGH images. It was thought that this was probably caused by the difference of cell mass in a certain volume between the two different kinds of tumour.

In the first part of the PEP study Faulkner's protocol (Faulkner et al., 1998) was used which involved modified cycles (50cycles to 40 cycles) with more segmented temperature cycles and 1/10 amount of primer. They suggested that reducing the oligonucleotide concentration to one tenth of its original decreased both the level and number of additional nonspecific bands in subsequent PCR amplifications without causing any marked loss in product level, and decreasing the number of thermal cycles from 50 to 40 also obtained a similar result. However Dietmaier et al (1999) have reported that the saturated amplification plateau was not reached before about 50 cycles with only two target copies and that reduction to 40 cycles resulted in false negative results. In the present study, even a primer concentration of 100 pmol showed no difference in yield

compared with higher concentrations and showed some decrease in the amount of high molecular weight DNA remaining in the well. A significant improvement was found when the number of thermal cycles was increased to 50 cycles and this was continued for the remaining experiments.

Cell lysis proved to be another critical step influencing the preamplification efficiency. In paraffin-embedded tissue, proteinase K is capable of degrading nuclear proteins involved in chromosomal packaging (histones) and thereby allows more efficient extraction of DNA. By reviewing results according to different cell lysis protocols in this study, it appears that proteinase K treatment might be especially important in fixed tissue where DNA packaging proteins are covalently cross-linked with each other or with DNA/RNA. Especially in small samples a high dose of proteinase K (1-2 mg/ml) and a short incubation ($\leq$ 24 hrs) produced good results.

From the observation of the quality of the CGH results from PEP amplified DNA produced by methods 1 to 7, it appeared that success depended not upon the PEP method itself but on the amount of starting DNA, the enzyme used, and the method of cell lysis.

In method 11, one aliquot of PEP product was further amplified by DOP-PCR and the CGH results using this DNA were compared with those produced by the original PEP product. The single PEP product showed very homogeneous images with true losses and gains of DNA copy number including losses of 1p, 5, 11, 15 and gains of 1q and 12p with prominent heterochromatin-rich area (centromeric regions on chromosome 1 and 9). By contrast, CGH results with PEP followed by DOP-PCR showed under-represented G-dark band regions and over-represented chromosome 19 and some telomeric regions. This discrepancy is thought to be caused by different characteristics of the primers. Also PEP followed by DOP-PCR has a risk of decreasing the reliability because too many replication cycles are performed. Products amplified by PEP + DOP-PCR showed a discrete band in the gel. This was thought to be caused by non-specific amplification of some portion of the DNA sequences by DOP-PCR amplification.

An interesting question, discussed in several papers, is whether PEP or DOP-

PCR amplifies the template DNA more precisely. Generally, PEP is the more accurate amplification method but cannot amplify the original copies to the same extent. Zhang et al (1992) described the advantage of the PEP protocol having little chance of mutation due to random amplification without any preference for specific sequences and similar chance of primer-extension product being involved in primer extension at a subsequent cycle.

By observation of the CGH result using product amplified by each method, there are some discrepancies in some regions such as 1p32-pter and G-light band areas on chromosome 5 and 11 in method 11. The above regions were under-represented by PEP in comparison with balanced state by DOP-PCR. This was thought to be caused by that regions of true loss were concealed by the over-represented nature of DOP-PCR on those areas. It is suggested that DOP-PCR preferentially amplifies G-C rich regions to a greater extent than PEP and PEP preferentially amplifies heterochromatin rich areas than DOP-PCR. In this study, molecular tests to prove this also had a problem because this amplified product was from DNA obtained from a whole section of tissue containing normal surrounding cells. Microsatellite instability and in particular detection of LOH require a homogeneous population of tumour cells to avoid a false negative LOH result due to any contamination by surrounding normal cells.

In this study, HMW product near the well appeared in almost all PEP experiments with the exception of methods 3, 9, and 11 which went on to produce relatively successful CGH results. It is certain that the success of CGH with PEP amplified product is closely related to HMW product in the well. The nature of this byproduct is truly unknown. In this study, it did not come out from the well in gel running with bigger ladders and the calculation of the ratio of spectrophotometric absorbance (A260/A280), which was around 1.8, can rule out contamination with cross-linking protein. By observation of the fact that failed cases showed a very faint smear, but with prominent HMW product, true effective fragments for the CGH experiment were at a very low concentration because efficient amplification and labelling were disrupted by this HMW product which might consumed enzymes during PEP amplification

or during nick translation. This is confirmed by the fact that although efficiency of nick translation of PEP product was much better than that of PCR labelling with DOP-PCR product, the success rate of the CGH experiments with DOP-PCR product was far better than those with PEP product.

In fact, although CGH was attempted many times with PEP product it was very difficult to obtain a successful result. So, to improve the efficacy and fidelity of PEP, the enzyme was changed from AmpliTaq LD to EHF PCR System and the later number of cycles was increased to 50. CGH results with PEP product using EHF PCR system showed improved quality of CGH in DNA from normal blood and abnormal bone marrow samples but not DNA from paraffin-embedded tissue.

Although the PEP method has proved to be applicable to various molecular studies, successful CGH using PEP product is very hard to achieve because of low degree of amplification ability.

Another problem of PEP amplification was frequent positive smear in negative control samples. This happened even when the same reagents, enzymes, pipettes and tubes were used as in DOP-PCR amplifications which showed no contamination. In this study, amplified negative controls with positive smear were used for CGH on several occasions and the results showed a balanced CGH image without any gain or loss. By comparing other PCR methods including DOP-PCR, PEP is seems to be more vulnerable to contamination by tiny amounts of DNA. This is thought be caused by the totally degenerate nature of primer.

4.8 CGH with serially diluted samples

After the publication of CGH results obtained from paraffin-embedded tissue by Speicher et al (1993), many CGH studies have been performed using product amplified from various sources of DNA ranging from microdissected paraffin-embedded samples to single cells from blastomere (Kallioniemi et al.,

1992; Speicher et al., 1993; Wells et al., 1999). Even if the amplified products contain preferential amplification and replication error to some extent which have been revealed by molecular genetic study (Keohavong and Thilly, 1989; Cariello et al., 1991; Cheung and Nelson, 1996; Sanchez-Ces-Pedes et al., 1998), CGH in association with WGA can become a valuable method to detect genomic changes in prenatal field and in cancer genetics if the fidelity and efficacy of the WGA method and reliability of CGH analysis could be increased.

The aim of this part of the study was to determine the smallest amount of template DNA which could be amplified to produce successful images.

In DNA from normal female blood, PEP technique could obtain a result only at 40 ng level of template DNA (approximately 4000-6000 cells). The best CGH results using amplified by PEP were achieved when PEP was carried out using EHF-PCR system and the same amount of normal control DNA was amplified by the same method. At the level below 40 ng of template concentration, CGH showed a disproportionately high level of hybridization to centromeric regions and very weak intensity of green signal elsewhere on the chromosomes. This finding was different from the results with DOP-product, which rarely showed over-representation in centromeric regions. This suggests three possibilities: first, regions of highly repetitive sequences were preferentially more amplified than other sites of chromosome at low concentration of template DNA: second, during hybridisation, probes fragmented into small pieces were reannealed first to highly repetitious sequences according to Cot curve: third, incomplete suppression of interspersed sequences by unlabelled Cot 1 DNA was an another possible explanation. The first assumption is more feasible than the others according the observation of several results. Wells et al reported that they failed to get any accurate results from DNA amplified using the PEP technique at the single cell level (Wells et al., 1999). Dietmaier et al. (1999) also reported that at least 10 unfixed cells from fluorescence- activated cell sorting, 10 cells of fresh frozen tissue and at least 30 cells from paraffin-embedded tissue were needed for reliable microsatellite analysis of PEP- amplified DNA. By considering Dietmaier's result which was performed with products with additional 50 cycles

of PCR amplification, CGH with PEP- amplified DNA might require over several thousand cells.

In DOP-PCR amplification in serial dilution of normal template DNA, CGH showed reliable results which appeared within normal range of fluorescence ratio profiles with the tendency of under-represented chromosome 1q32-pter, 16, 19, and 22 above 75 pg level of template DNA concentration. By contrast at or below the 50 pg levels, these regions appeared disproportionately over-represented. Also G-dark band regions showed much weaker green signals than other parts of the chromosomes. These findings suggest that regions rich in GC with CpG islands are preferentially amplified at very low concentrations of template DNA even though hybridisation preference also cannot be ignored. In this study, specific band around 400 bp on agarose gel was frequently found. This was thought to be caused by non-specific amplification of some portion of the DNA sequence.

Cheung and Nelson (1996) suggested that the DOP-PCR product cannot represent the whole sequence of the starting genomic DNA and usually amplifies one third as well. They suggested three possible explanations about DOP-PCR amplification: I) production of nonspecific DNA created by non-specific priming; ii) over-representation of highly repetitive DNAs by the PCR process: iii) production of overlapping sets of DNA fragments. The results of this experiment can be seen as proof for their first and second explanations.

Then, to increase the reproducibility and reliability of CGH results, the proofreading enzyme (EHF PCR system) was used instead of thermosequenase or AmpliTaq LD and CGH was carried out by hybridising with reference DNA amplified and labelled by the same method. These CGH results showed quite uniform, evenly hybridized images and profiles with adequate sexing, and did not show disproportionately over-represented or under-represented regions even at the level of 10 pg (about single diploid cell) of template DNA. It is suggested that any irregularity or preferential amplification of samples during DOP-PCR and labelling procedure did not affect the CGH profiles produced using this method.

The system was then investigated using DNA from abnormal bone marrow

from a patient with AML /MDS. The same dilution method and WGA with PEP or DOP-PCR were applied. The aim of this experiment was to find how accurately the CGH results obtained using products amplified by PEP or DOP-PCR compared with the known CGH results of the starting samples.

By applying PEP, the results were nearly the same as that of a normal female blood sample. Only a concentration of 40 ng of template DNA, hybridised with normal reference DNA amplified by the same method, showed reliable CGH images.

In experiments with DOP-PCR, above 300 pg level, CGH results exhibited almost all the abnormalities known to be present but the intensity of signals became weaker as the concentration decreased. Also distorted findings, which were shown at the concentrations below 75 pg in normal sample, appeared from the concentration below 300pg of template DNA. But it became possible to obtain better results (3 out of 4 abnormal findings) even at 50 pg (about 8 cells) of template DNA by using proofreading enzyme and hybridising with normal reference DNA amplified simultaneously.

The third trial used DNA from thick sectioned paraffin-embedded tissue as template DNA. The original CGH result using DNA without amplification was not biased, so this experiment could evaluate the degree of coverage of abnormal findings by CGH using amplified product from paraffin-embedded tissue.

By PEP amplification, no successful result was obtained. This means that DNA from paraffin-embedded tissue was more fragmented than that from other samples and so the amount of optimally sized DNA fragment for CGH after amplification was lower than that of same concentration of other samples.

In this experiment using DOP-PCR, 6-7 out of 7 abnormalities in gain and 5 out of 7 abnormalities in loss were identified above 500 pg level. Losses of DNA copy number were less accurate than gains. The 500 pg level was a turning point below which unreasonably over-represented regions on 1p32-pter, 19, 20, and 22 were observed. After experiments using proofreading enzyme and hybridizing with normal reference DNA amplified simultaneously, gains were reasonably well detected at concentrations above 100 pg (100% at 200 pg,

85.7% at 100 pg) but losses were less distinctive (71.4% in 200 pg, 57% in 100 pg). Below 50 pg, the result was not meaningful but did not show up disproportionately over- or under-represented regions.

In CGH, usually copy number increase is visible if the gained region is 2 Mb or larger. Resolution for deletion of 5-10 Mb might reasonably be accepted in practical experiments because of several factors, such as varying condensation of the target metaphase chromosomes, intratumour heterogeneity and contamination with non-tumour cells (Piper et al., 1995). Therefore, in experiments with paraffin-embedded tissue, resolution may be further decreased.

In conclusion, CGH using products of WGA can provide reasonable results above a certain level of concentration, which is dependent on the quality of the original samples. Reliable CGH can be obtained above 10 pg (about a single diploid cell) of DNA from normal female blood, above 50 pg (about 8 cells) of DNA from abnormal fresh sample and above 100 pg (about 15 complete cells) of DNA from paraffin-embedded tissue when amplification is performed with EHF PCR system as an enzyme and the normal control DNA is amplified and labelled by the same method as test DNA.

4.9 CGH experiments on very small amounts of DNA in solid tumours

Genetic study in solid tumours has developed quickly through the introduction of various whole genome amplification methods which enabled studies using very small amounts of DNA and also the use of archival samples such as paraffin-embedded tissue. Even though the DNA is already fragmented or still intermingled with nucleoprotein, the development of cell lysis methods, proper adjustment of DOP-PCR thermal cycles and development of new enzymes have made it possible to produce adequate amounts of DNA for CGH from very small amounts of microdissected materials from paraffin-embedded tissue.

Although there are an increasing number of papers describing CGH using WGA with microdissected samples, the minimal requirement of DNA to achieve reproducible CGH with minimal artifact, and method for improving the fidelity of CGH results from paraffin-embedded tissue samples have not yet been systematically evaluated and optimised.

There are many critical points during the progression of this experiment. Sample status, cell lysis methods, thermal cycle condition, and enzymes used have already been discussed in chapter 4.4. From the point of view of diagnostic and prognostic significance of test results, the most important factors are the reliability of WGA and the minimum amount of template DNA required to avoid serious artifact (false positive or negative results).

To increase the reliability, it is important that as little non-tumour tissue as possible is present. Combining whole genome amplification methods with novel laser based microdissection techniques has made it possible to decrease contamination with non-tumour cells including normal epithelial cells, stromal cells, endothelial cells, and inflammatory cells (Emmert-Buck et al., 1996; Bohm et al., 1997).

In the first part of these experiments where DNA was extracted from one whole section of 7 μm thickness of paraffin-embedded melanoma samples, it was possible that the tumour DNA was contaminated with non-tumour DNA, and consequently the CGH results with amplified product from these samples reflected the average content of all cell types present in the samples. Although CGH can detect abnormalities from samples with below 50% contamination of non-tumour cells, there are some limitations in accepting this level in paraffin-embedded tissue samples because of intratumour heterogeneity in addition to contamination with normal cells.

In this study, one sample was the subject of three different kinds of experiments (using whole section and microdissected materials with two different enzymes). By comparing the three findings, the result from whole sections of 7 μm thickness showed a less accurate detection of abnormal findings especially loss of DNA copy number (3 out of 3 in gains, 1 out of 5 in losses); on the other

hand, non-specific findings were much increased. These non-specific findings were similar to findings observed when the concentration of template DNA was extremely low. This suggests that experiments with whole sections may not be able to detect all DNA copy number changes by CGH because true abnormal findings are diluted with surrounding normal tissue and this dilution will make it more difficult to produce adequate amount of tumour DNA amplification for CGH.

Microdissections were then performed on subsequent unstained slides under the inverted microscope using 25G sterile needles according to regions of tumour cells identified from Haematoxylin-Eosin (H-E) stained slide. H-E staining may not be desirable for microdissection because of the adverse reaction on DNA (Burton et al., 1998).

Another critical point in this experiment is that microdissected tissue sections can encounter a serious problem because the cell and nucleus volume is diminished by cutting. As a result, chromosomes or parts of chromosomes can get lost, leading to false loss of DNA copy number (Speicher et al., 1995; Duddy et al,.1998; Dietmaier et al, 1999). This is supported by the fact that control FISH experiments with centromeric probes on normal tissue sections showed loss of chromosomes in 15-20% of all nuclei (Speicher et al., 1995). To overcome this problem, larger areas should be microdissected from thinner cut samples.

In this study, microdissected areas were approximately $2mm^2$ x 3 μm, $9mm^2$ x 3μm, $18mm^2$ x 3 μm, $9mm^2$ x 7 μm. A microdissected volume of $2mm^2$ x 3 μm could not yield an adequate amount of amplified DNA to produce proper CGH images. Then microdissected material was increased to 18 mm^2 x 3μm and successful results were obtained in all cases. At a volume of 9 mm^2 x 3μm section, all cases except one showed DNA below 500 bp in size (mainly below 300 bp) and failed to produce CGH images. However one case, which showed 300 to 1000 bp sized fragments, obtained reasonable CGH images at the same size of microdissected area by WGA with AmpliTaq LD and EHF PCR system. The three failed cases were repeated using increased volume of the tumour cell

mass (9 mm^2 x 7 μm) and reasonable results were obtained. The one remaining case procured only 3 mm^2 x 7 μm because of the very small size of the original tumour mass and an adequate result could not be obtained. These results suggest that amount of DNA to be extracted may depend on the difference of cell mass inside the tumour and the quality of paraffin-embedded tissue in the same size of microdissected volume. The critical factors for successful CGH are also not only the sample size but also size of DNA fragments after amplification.

This study also evaluated the efficacy and reliability of three kinds of enzyme, namely AmpliTaq LD, Pfu DNA polymerase, and EHF PCR (Expand High Fidelity PCR) system. Although the result with Pfu DNA polymerase was more reliable than that with AmpliTaq LD, overall findings were very similar especially losses which showed a completely identical result. If Pfu DNA polymerase had been more optimized, it would probably have given a better result. Comparing AmpliTaq LD to EHF PCR system, the results with EHF PCR system were superior from the point of view of reliability and reproducibility (more amplified and bigger DNA fragments) than those with AmpliTaq LD. Above a certain level of template DNA, there was no significant difference of results between AmpliTaq LD and EHF PCR system but in very low concentrations of template DNA, experiments with EHF PCR system showed more sensitive and reliable results. Experiment with AmpliTaq LD showed improved results when both test and reference DNA were amplified simultaneously by reducing the noise difference but revealed low hybridization intensity. Although proofreading enzyme also affects the efficiency and fidelity of whole genome amplification, the degree of fragmentation of DNA in paraffin-embedded tissue and the amount of true tumour cell mass in microdissected sample are more critical factors than the enzyme in CGH with universal whole genome amplification.

Finally, this experiment was to assess whether PEP can amplify sufficient amount of product for successful CGH in melanoma samples.

By PEP application, all cases except one showed large amount of high

molecular weight DNA near or in the well and all of these cases failed to amplify an adequate amount and proper quality of DNA for CGH. One case, which did not show up high molecular weight DNA, obtained successful CGH images. Once again, high molecular weight DNA near or in the well has a negative effect on amplification process, labelling process, and CGH. The most annoying problem during PEP amplification was positive smear in a negative control sample.

The conclusion was that PEP is not a suitable amplification method for producing reliable CGH images in this amount of tumour cell mass even though many papers have said that PEP offers more complete coverage at lower genomic DNA copy for microsatellite instability assay or loss of heterozygosity (Dietmier et al, 1999). The cause of failure might be too low concentration of amplified product for CGH and decreased efficiency due to consumption of primer and enzyme by non-specific HMW product.

DOP-PCR amplification obtained reliable CGH results according to the amount of suitable size of DNA, and enzyme used. During this experiment, the investigation of how much the sequence of template DNA can be covered by DOP-PCR was continued because this directly affects the reliability of CGH results. Based on the number and sizes of DOP-PCR products, theoretically there should be about one million DOP-PCR fragments generated from the entire human genome because DOP-PCR primer probably can anneal every 4 kb. If the average 500 bp sized product is produced in the haploid human genome (about 3x 10^9 bp), it is possible to anticipate that an arbitrary stretch of DNA had only a one in six chance of being included in the DOP-PCR product (Cheung and Nelson, 1996). By considering the capability of detection of CGH (average 3-5 Mb), this does not affect the CGH result itself. The limitation of spatial resolution of CGH is already documented in that deletions detected by LOH or FISH were not diagnosed by CGH in several studies (Cher et al., 1994; Joos et al., 1995; Benz et al., 1998).

After several trials of CGH experiments with DOP-PCR amplification product, relatively consistent results were obtained, although there were some discrepancies according to sample size and enzyme used. These results showed

gains on 6p, 15q and losses on 9p, 10p, 6q, 17p which are known to be areas frequently involved in melanoma. But areas frequently involved in fresh frozen samples studied here, such as gains on 7, did not appear in this part of the study and also in contrast with loss of 10q which was a more prevalent finding in fresh frozen samples, 10p involvement was more prominent in the work with paraffin sections. By Bastian et al. (1998), a gain of chromosome 7 was found more frequently in thicker lesions. This finding suggest that these samples were in relatively early stage of melanoma. Because no information about patient status was available during these studies, it is not known whether these discrepancies are due to the stage of the tumour or are an experimental artifact. The small number of samples studied also make it difficult to draw any conclusion.

In conclusion, even if CGH results with DOP-PCR products showed some unwanted over- or under-represented regions, DOP-PCR was found to be a better method of amplification for producing CGH images than PEP in CGH experiment with very small amount of DNA and the minimal requirement of tissue size is over 3mm x 3mm x 3μm in melanoma case. A proofreading enzyme such as EHF PCR system is considered to be the enzyme of choice. CGH with WGA can become a useful method to detect true genomic copy number changes and to evaluate genomic progression from premalignant lesion to metastatic tumour by developing the techniques of WGA.

4.10 Validation of CGH results

The main problem in the amplification of CGH with product amplified using WGA is the reliability of the result. During the amplification process, many artifacts developed due to high order structure of DNA and varying degree of amplification error of enzyme. Generally, estimate of the error rate (error/bp per duplication) of Taq polymerase is 2×10^{-4} / bp per duplication. A T to G C transitions with Taq polymerase frequently occur (Dunning et al., 1988:

Cariello et al., 1991).

In order to prove the fidelity of DOP-PCR products, it was important to examine how uniformly DOP-PCR amplifies the sequences of template DNA. Inclusion of a normal DNA sample in every DOP-PCR reaction batch is very important to verify the CGH result. Only if the green to red fluorescence ratio of all chromosomes remains in the normal range in the control sample, unknown test samples can be reliably analysed (Kuukasjärvi et al., 1997).

Another validation option is a molecular method such as microsatellite instability (MSI) and loss of heterozygosity (LOH). MSI analysis, and in particular detection of LOH, require a homogeneous population of tumour cells to avoid erroneous underestimation of the LOH frequency due to any contamination by adjacent normal cells (Dietmaier et al., 1999). In this study, MSI or LOH did not perform because the DNA to be amplified was thought to be contaminated with normal surrounding cells in most of the experiments with melanoma sections and it was not possible to obtain blood or normal tissue sample from the patient as a control. Therefore these amplified products were subjected to PCR with specific primers (a 30 mer derived from pseudoautosomal sequences in combination with 30 mer from X-specific and Y-specific regions, exon 2 of *p16* on chromosome 9p21). In the PCR results for sex determination, all melanoma samples failed to show any band in comparison with compatible bands of male and female controls. This finding indirectly suggests that all melanoma samples were already fragmented below 771 bp. In the PCR results with primer of exon 2 (*p16*), normal control showed a single band at 337 bp. Among five melanoma samples, only case 3 showed a single faint band at 337 bp. From these results, it is not sufficient to reach a definite conclusion. This experiment needs more time for optimisation and quality control.

FISH to metaphase chromosomes using probes for areas of interest and tissue in situ hybridization is a possible method of validation. FISH to metaphase chromosomes could be rarely performed because of less availability of metaphase chromosomes in solid tumours. Tissue in situ hybridisation also had some difficulties arising from loss of chromosome material by cutting, three-

dimensional structure of interphase nuclei, and in discrimination between normal and malignant cells under the DAPI staining. The results showed inconsistent findings because of overlapping and biased false negative signals. As a validation method, tissue in situ hybridisation on paraffin-embedded section showed a possibility in spite of several drawbacks such as difficulties in obtaining appropriate probes and in counting signals. For improvement of this technique it is recommended to use confocal microscopy for counting and increase the availability of probes for specific locus interested.

Inverse CGH was the simplest method for validation of results. Larramendy et al (1997) found that when Texas Red was used to label test DNA, over-representation was observed at 4q13-q21, 11q21-23, 13q21-qter, and Xq21-q22, whereas 19p was under-represented. These findings were not observed in this study. By considering the difference between two kinds of CGH profile according to the ordinary and inverse labelling, CGH with DOP-PCR products using template DNAs from melanoma samples showed relatively compatible results. In cases 1 and 2, CGH results could not be sure because quality of original DNA was extremely poor.

In conclusion, CGH can be an important complementary method to conventional karyotyping in hematologic malignancies.

In CGH with serially diluted DNA, the results showed the smallest amount of template DNA for reliable CGH- 10 pg (about single diploid cell) from normal female DNA, 50 pg (about 8 cells) of DNA from abnormal fresh bone marrow, and 100 pg (about 15 cells) of DNA from abnormal paraffin-embedded tissue. From these results, if the purpose of study is for the detection of numerical abnormalities, reliable CGH results from a single cell can be achieved when amplification is performed with EHF PCR system as an enzyme and the same amount of normal control DNA is treated by the same method as the test DNA.

In the case of tumour and in particular melanoma, where cytogenetic studies are difficult, this study has shown how methods can be improved to yield good quality and reproducible CGH images. It is likely that CGH studies will now be able to detect important changes in the early stages of tumour formation, to

follow these during development from formalin fixed sections and to identify new amplicons where might be another possible region to search for new candidate oncogenes.

Further improvements might come from use of laser based microdissection technique, use of control material from normal tissue on the same slide and development of enzymes with high fidelity. The use of touch preparations in the early stages would also be of great benefit. It was hoped to include this approach in the present study but no suitable cases presented in time.

CHAPTER 5. REFERENCES

5. REFERENCES

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