N-ACETYLTRANSFERASE 2 IN THE RECURRENCE AND PROGRESSION OF PATIENTS WITH BLADDER TRANSITIONAL CELL CARCINOMA

A thesis submitted to the University of Glasgow for the degree of Master of Science in the Faculty of Medicine

by

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Based on research conducted in the University Department of Surgery, Glasgow Royal Infirmary

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DEDICATION

This thesis is dedicated to my dear family, who have been supportive and encouraging throughout, and without whom this work would not have been possible.

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DECLARATION

This thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

SUMMARY

N-acetyl transferase 1 and 2 are metabolic enzymes involved in the detoxification or bioactivation of aromatic amines. The development of transitional cell carcinoma of the bladder is associated with exposure to chemical carcinogens, either from occupational exposure or through smoking, and many chemical carcinogens are aromatic amines. The genes that encode the *N*-acetyl transferase enzymes are polymorphic, and particular alleles are associated with an increased incidence of bladder cancer.

Transitional cell carcinoma of the bladder is the fourth commonest cancer in the UK and USA. Repeated recurrences (in up to 80% of patients), place an individual at increased risk of developing muscle invasion. However it is not possible to predict, from clinical and pathological data, which index (primary) carcinomas will recur. Due to the increased risk of recurrence and progression of non-muscle-invasive transitional cell carcinoma, patient management involves prolonged clinical follow-up as at present there are no reliable markers to predict the biological behaviour of an index, non-invasive transitional cell carcinoma.

Intensive scientific research over the past decade into genetic mechanisms involved in transitional cell carcinoma has established two distinct genetic pathways to progression, and recently markers of recurrence have also begun to emerge.

The aim of this study was to use a DNA probe for the *N*-acetyl transferase 2 gene (*NAT2*) and to investigate whether genetic abnormalities at this locus contribute to recurrence or progression in an index, non-muscle-invasive transitional cell carcinoma, and whether patients who present with muscle-invasive transitional cell carcinoma (stage pT2 and above) have an abnormal genotype. *NAT2* is on chromosome 8p, a region commonly aberrent in many carcinomas including bladder. In order to achieve this aim, it was necessary to obtain a cosmid containing the *NAT2* sequence, label the cosmid to produce a probe for fluorescence *in situ* hybridisation, and then to apply the technique to formalin fixed paraffin processed sections of transitional cell carcinomas. This process proved technically challenging, but once the methodology was working consistently well on formalin fixed paraffin processed control material, sections of transitional cell carcinomas were assessed in a dual labelling technique with the *NAT2* probe and an alpha centromeric probe for chromosome 8.

Nineteen patients were assessed, with full clinical follow-up (median=55 months), in eight of whom carcinomas recurred. Fluorescence *in situ* hybridisation was also applied to the recurrences, and a total of 37 carcinomas were assessed.

Of the 19 primary carcinomas assessed, five were muscle-invasive at presentation (stage pT2 or above), the remainder were stage pTa or pT1. All five muscle-invasive carcinomas had polysomy of chromosome 8, four of whom also had abnormal *NAT2* copy number. Another six primary carcinomas (all stage pTa or pT1) had abnormal chromosome 8 and/or *NAT2* copy number. Of the 18 recurrences, 9 were abnormal for chromosome 8 and/or *NAT2* copy number, eight of which were in patients who progressed at recurrence to muscle invasion (pT2 or above).

Overall, a high proportion of carcinomas 54% (20/37) when assessed by mean chromosome or gene copy number, were abnormal for either gene or chromosome copy number or both, and this was significantly associated with high grade, p=0.014 and stage pT2 or above, p=0.039. There were more abnormalities in patients with detrusor muscle – invasive disease.

Few studies of abnormalities of gene and/or chromosome copy number in transitional cell carcinoma of the bladder achieve full clinical follow-up, and are unable to relate their findings to the clinical course of the disease. The abnormalities of *NAT2* demonstrated in this study were more common in patients with muscle-invasion at some point in their disease history, linking abnormal function of *N*-acetyl transferase 2 with aggressive disease course. Polysomy 8 was also common, and abnormalities of chromosome 8 have been previously been associated with late stage progression in transitional cell carcinoma.

In conclusion, by studying the gene and chromosome copy number of *NAT2* and chromosome 8 in index transitional cell carcinomas and any subsequent recurrences further understanding of the role of metabolic enzymes in transitional cell carcinoma has been realised. For molecular biology to have any impact on patient management, studies such as this one, recapitulated with larger numbers, will begin to become a much needed additional tool in the accurate diagnosis and management of this complex cancer.

ABBREVIATIONS

TCC = transitional cell carcinoma of the urinary bladder

NR = non-recurrer, individual who presented with a single episode of non-detrusor-muscle-invasive TCC and no further recurrence, median follow-up 79 months (range 24-106 months).

RNP = recurrer-non-progressor, an individual who, following an initial presentation with non-detrusor-muscle-invasive TCC, subsequently recurred, but did not progress to detrusor-muscle-invasion, median follow-up 40.5 months (range 18-135 months).

RP = recurrer progressor, an individual who following an initial presentation with non-detrusor-muscle-invasive TCC, subsequently developed detrusor-muscle-invasion, median follow-up 37 months (range 14-152 months).

PP = progressed at presentation, an individual with detrusor-muscle-invasion at first presentation.

pTa = superficial TCC

pT1 = TCC locally invasive to the sub mucosa (lamina propria)

pT2 = TCC invasive to detrusor-muscle layers

NAT2 = the gene for the metabolic enzyme *N*- acetyl transferase 2

FISH = fluorescence *in situ* hybridisation

CHAPTER 1 INTRODUCTION & STUDY AIMS

1.1 Bladder Cancer

Bladder cancer is the fourth commonest neoplasm in the UK. The incidence of bladder cancer has risen 3-fold over the last 30 years (Office of Population Censuses and Studies, 1969-1987; 1971-1984), as illustrated in Figure 1. The causes of bladder cancer are multifactorial but exposure to chemical carcinogens, for example from smoking or as an occupational hazard, is thought to be particularly important (Sandberg & Berger 1994). Bladder cancer is the fifth commonest cause of deaths in the UK, 5800 die annually in England and Wales (Abdel-Fattah *et al*, 1998), 450 annually in Scotland (Scottish Cancer Registry, 1995).

1.1.1 Clinical Presentation

The bladder is a reservoir for urine and lies in the pelvic cavity; its size and position vary depending on the amount of urine it contains. The bladder wall is composed of three layers (illustrated in Figure 2). The outer layer, of loose connective tissue containing nerves, blood and lymphatic vessels, is covered on the upper surface by peritoneum. The middle layer of interlacing smooth muscle fibres and elastic tissue loosely arranged in three layers forms the detrusor muscle which empties the bladder when it contracts. The inner layer is a lining of transitional epithelium and subjacent connective tissue. When 300–400ml of urine have accumulated afferent autonomic nerve fibres in the bladder wall sensitive to stretch are stimulated. Micturition occurs when the detrusor muscle contracts, there is a reflex relaxation of the internal sphincter and voluntary relaxation of the external sphincter (Wilson & Waugh 1996).

The majority of bladder cancers (90%, Raghavan *et al*, 1990, Soloway, 1992) are transitional cell carcinomas. Much less commonly, squamous cell carcinomas (Cotran, 1987), adenocarcinomas (2.5%), sarcomas, melanomas, small-cell undifferentiated carcinomas or metastases from other primary tumours may occur in the bladder (Raghavan *et al*, 1990). Clinical appearance of transitional cell carcinoma varies from a single superficial, non-invasive low-grade papillary lesion to numerous or extensive highly malignant anaplastic tumours (Raghavan *et al*, 1990). The latter account for 20% of transitional cell carcinomas and have a poor prognosis (5 year survival 35%) (Knowles, 1995) as they tend to be detrusor-muscle-invasive. Fifty to 70% of non-invasive transitional cell carcinomas will develop recurrence (van der Meijden, 1998)

about 20% of which will progress to muscle-invasion (reviewed by Adshead *et al*, 1998).

1.1.2 Clinical Staging

Although the histogenesis of transitional cell carcinoma is unclear (Raghavan *et al*, 1990) a classification of urothelial tumours has emerged following TNM guidelines which gives a clinical and a pathological basis to the diagnosis (Sobin and Wittekind, 1997). The classification system is summarised in Figure 3 (van der Meijden, 1998).

This system of classification came about after repeated observations that survival and recovery rates were higher for patients with localised disease compared to those with invasive disease. Using the TNM classification, conformity between clinicians is achieved and appropriate treatment can be planned (Pavone-Macaluso, 1984, Droller, 1985), although T2 and T3 transitional cell carcinoma cannot be distinguished in cystoscopic biopsies. The T stages of bladder tumours are illustrated in Figure 4 (Cotran, 1987).

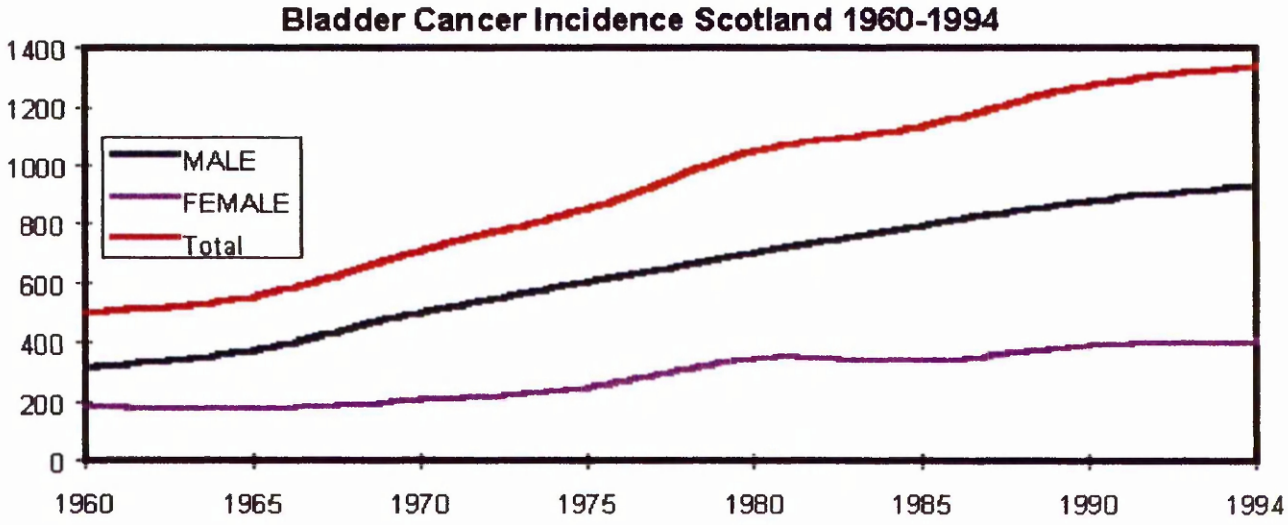
1.1.3 Pathological Staging

Pathological examination of a diagnostic bladder tumour biopsy covers three stages. First it must be established that the lesion is truly neoplastic, and the cell type identified. As indicated above, the commonest bladder tumours are transitional cell carcinoma, with occasional metaplastic squamous or glandular areas. True squamous carcinomas are rare in the West but are the commonest bladder cancers in Egypt due to urinary schistosomiasis (Bedwani *et al*, 1998).

Secondly, the lesion is graded using TNM guidelines *i.e.* grade 1, well differentiated; grade 2, moderately differentiated; grade 3/4, poorly/undifferentiated (Sobin and Wittekind, 1997).

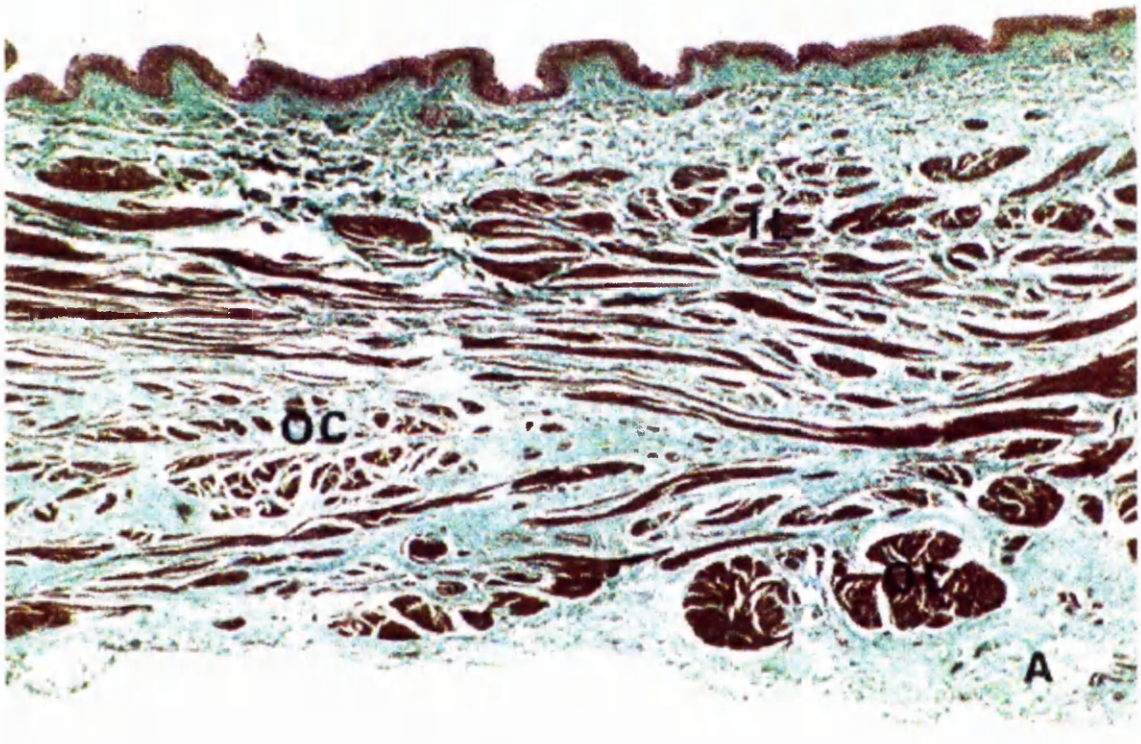
Finally, the stage is assessed as far as possible based on observations of the overall growth pattern and the presence or absence of invasion by the lesion *i.e. in situ* pTis, papillary non invasive pTa, papillary invasive pT1, papillary and solid pT2 and, where the true extent of invasion cannot be assessed, pTx. The prefix "p" is used to denote a pathological diagnosis, as opposed to the "T" classification, without the "p" denoting the clinical diagnosis.

Figure 1: Incidence of Bladder Cancer in Scotland



This graph illustrates the increasing incidence of bladder cancer in Scotland

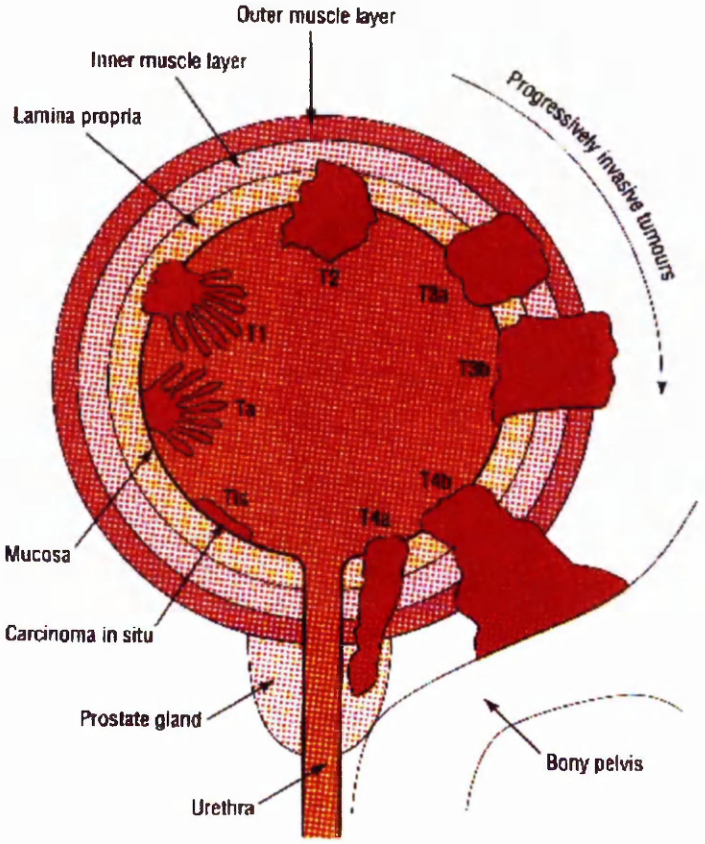
Figure 2 The Structure of the Bladder Wall



IL = inner longitudinal smooth muscle layer; OC = outer circular smooth muscle layer

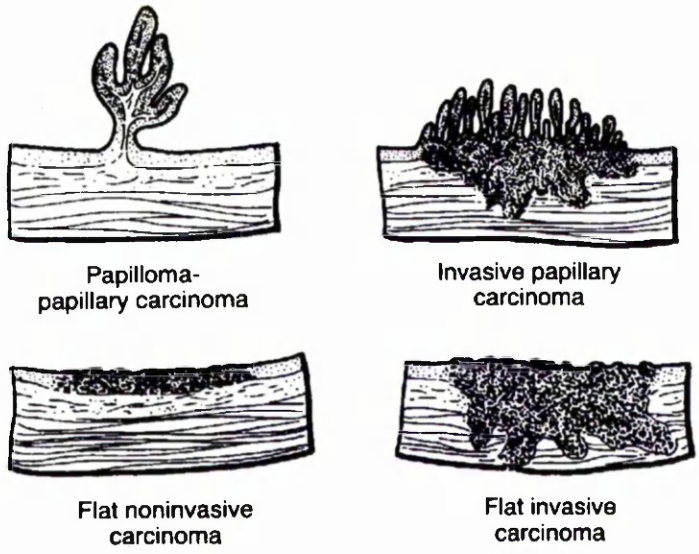
OL = outer longitudinal smooth muscle layer; A = adventitia

Figure 3 Tumour Staging According to the TNM System, 1997. (reproduced from van der Meijden, 1998).



A method of classification of urothelial tumours which involves staging, depending on the degree of invasion. Ta=non invasive papillary; Tis=flat carcinoma *in situ*; T1=locally invasive to the submucosa (lamina propria); T2=invasive of the muscularis; T3a=beyond muscularis microscopically; T3b=extra vesicular mass; T4a=infiltration of prostate, uterus or vagina; T4b-fixation to the pelvis or abdominal wall (Sobin & Wittekend, 1997).

Figure 4: The Four Morphological Patterns of Bladder Cancer



This diagram illustrates the main features of the four commonest forms of bladder cancer

1.1.4 Treatment and Patient Management

Eighty-five percent of transitional cell carcinoma patients present with gross or microscopic haematuria at some point but this is intermittent, even when caused by muscle invasive transitional cell carcinoma (Goldstein & Messing, 1998). But because of the low specificity and pick-up rate, despite the high sensitivity, haematuria is not used as a screening test for the presence of transitional cell carcinoma. Repeated tests would need to be carried out to enable a diagnosis of transitional cell carcinoma to be made (Goldstein & Messing, 1998). Urgency and frequency may also reflect the presence of carcinoma *in situ* (Droller, 1985).

Bladder cancer is commoner with advancing age, and older patients have more poorly differentiated, invasive tumours than younger patients (Anderson, 1982). Increasing incidence with age is well established but not fully understood, although several genetic aberrations are required for a tumour to become fully malignant (Nowell, 1976). The latency associated with exposure to environmental carcinogens and the development of cancers, for example ≥15 years for asbestos and the development of mesothelioma (Attanoos & Gibbs, 1997) and up to 40 years for bladder cancer (Risch *et al*, 1995) supports this paradigm.

Three times as many men as women develop bladder cancer, a phenomenon that has been known for many years (Case *et al*, 1954, Raghavan *et al*, 1990). The reasons for this are unclear, although the majority of cases have been attributed to environmental exposure in industries predominantly occupied by men. In addition, the exposure to many carcinogens in developed countries is now strictly limited by industrial health and safety legislation.

Urine cytology, the simplest and least invasive diagnostic procedure, has a poor record for detecting malignant cells. Low grade carcinomas are less likely to to shed cells, due to the low rate of cell turnover. A higher percentage of positive samples are observed with grades 2 and 3 transitional cell carcinoma. Despite its poor sensitivity, urine cytology is used in occupational screening of workers at high risk of occupational bladder cancer. Results have been encouraging with a higher pick-up rate of positive cytology than in the general population (Droller, 1985).

Bladder washings increase the diagnostic yield possibly because low-grade tumours may shed papillary fronds through this procedure (Droller, 1985), increasing the

detection rate for low-grade carcinomas. Cystoscopy is the gold standard for diagnosis, allowing a direct view and biopsy of the mucosa (Steiner *et al*, 1997).

1.1.5 Treatment and Follow-up of Superficial Transitional Cell Carcinoma

Initial treatment of superficial and superficially locally invasive carcinomas (pTa, pT1 and carcinoma *in situ*) is usually by transurethral resection (Holmäng *et al* 1995), diathermy or both (Raghavan *et al*, 1990). This is followed by surveillance cystoscopies, usually 3 monthly for the first year, 6 monthly for the second year and then annually (Tolley *et al*, 1996), unless there is a recurrence in which case the above sequence is repeated. After several recurrences, especially if the tumours become muscle invasive, cystectomy may eventually be performed. Cystectomy may be required earlier in cases of carcinoma *in situ,* a high grade lesion entirely different to non-invasive papillary transitional cell carcinoma.

Reducing the recurrence of transitional cell carcinoma and thus the number of cystoscopies due to its high prevalence is the aim of several researchers, who acknowledge that this would reduce patient trauma and cut down the resources allocated to management of transitional cell carcinoma. Holmäng and co-workers(1995) argue that patients with low-grade superficial carcinomas *i.e.* pTaG1 treated by transurethral resection of the tumour are at low risk of recurrence and are effectively cured by this procedure. But in patients with frequent recurrences of multifocal carcinomas, which carry an increased risk of progression to muscle invasion relative to patients with non-recurrent disease, Holmäng and co-workers (1995) suggested earlier and more aggressive treatment, *i.e.* with intravesical chemotherapy or cystectomy. With the conventional management of superficial transitional cell carcinoma, *i.e.* transurethral resection of the tumour and/or diathermy or cystectomy, long-term survival of patients with superficial transitional cell carcinoma has been achieved in 80% of cases, but complete cure rate remains at less than 50% of all cases diagnosed (Raghavan *et al* 1990). These statistics have remained constant over the past ten years (van der Meijden, 1998).

Chemotherapy of superficial transitional cell carcinoma has included intravesical chemotherapy, in which cytotoxic agents are instilled by a urinary catheter directly into the bladder, has been more widely applied over the past 20 years than previously (Raghavan *et al,* 1990, Holmäng *et al,* 1995, Tolley *et al*, 1996). Prolonged disease-free

interval after transurethral resection of the tumour was observed if agents such as thiotepa, eoglucid, doxorubicin or mytomycin C were administered (Raghavan *et al*, 1990). A large randomised trial (Tolley *et al*, 1996) demonstrated decreased recurrence rates and increased disease free intervals (decreased recurrence risk of 15%) after intravesical mytomycin C was administered for newly diagnosed superficial bladder cancer, with follow-up over seven years. There appeared to be no significant benefit to giving repeated instillations.

In a separate trial, by the Genito-Urinary Group of the European Organization for Research and Treatment of Cancer, a single instillation of epirubicin was effective at decreasing recurrence (nearly 71% of treated patients were free of recurrence after 2 years compared to the 59% in the control group), and that recurrence rate was approximately halved by use of a single installation of epirubicin (Oosterlinck *et al*, 1993).

An alternative treatment involves the administration of the BCG (bacillus Calmette-Guerin) vaccine. This form of immunotherapy appears to be most effective in carcinoma *in situ* (Raghavan *et al*, 1990). A comparison between BCG and doxorubicin for carcinoma *in situ* showed remission in 74% of cases treated with BCG as opposed to 42% treated with doxorubicin, with a median disease free interval of 48 months for BCG treated patients, 5.9 months for doxorubicin, (Hudson & Herr, 1995). Immunotherapy rather than cystectomy has been recommended as the initial treatment for CIS (Hudson & Herr, 1995).

At present clinical management of either superficial transitional cell carcinoma or carcinoma *in situ* involves repeat cystoscopies with transurethral resection of the tumour that may incorporate diathermy followed by instillation chemotherapy or immunotherapy and/or cystectomy.

1.1.6 Treatment and Follow-up of Muscle-invasive Transitional Cell Carcinoma:

The two classic ways to treat muscle invasive disease are surgery and/or radiotherapy. Radical cystectomy (involving excision of the bladder and contiguous organs and tissues as a block) is associated with improved survival rates over simple cystectomy (where only the bladder is removed). Actuarial five year survival rates of 35% to 75% have been reported following cystectomy and lymphadenectomy (Raghavan

et al, 1990). The higher survival rates were observed in patients with disease confined to the bladder and contiguous organs. Radiotherapy has been less widely used in the past 30 years compared to in previous years, but with the recent development of precise delivery of radiation through computerised tomography scans, this treatment modality has been revived.

Despite improvements in surgical and radiotherapy techniques, survival of patients has not changed. Up to 50% develop metastases and die within 5 years (reviewed by van der Meijden, 1998), and consequently other approaches in the management of advanced TCC have been investigated. Neoadjuvant chemotherapy whereby the chemotherapeutic agent is administered before surgery or radiotherapy may confer benefits such as reducing tumour bulk and minimising micrometastatic disease, although patients must be in good health otherwise before beginning this type of treatment. With this approach, 5 year actuarial survival rates of 35%-70% have been reported (reviewed in Raghavan *et al*, 1990) and quality of life has been improved.

1.1.7 Pathology

Disparities exist in the pathological reporting of bladder carcinomas, with high inter- and intra-observer variability and inconsistency in the assessment of clinically important data. When bladder tumours were reassessed seven months later by the same group of pathologists who had made the original diagnosis, there was inconsistency of reporting WHO grade in almost 50% of cases (Ooms *et al*, 1982). Potentially important considerations are raised as grade 1 and grade 3 carcinomas will be treated differently. Although there does appear to be wide variation in the consistency of reporting of bladder tumours, a higher percentage of conformity was observed by Witjes and co-workers (1994) than the study by Ooms and co-workers (1982) with figures of 79.3% for stage and 70.2% for grade. They claimed that although review of pathology caused considerable changes in the results, there was no change to treatment and virtually no difference in the results of a prognostic factor analysis. There remains controversy over these inconsistencies and additional work is required to address this problem.

1.1.8 Summary

Bladder cancer, in particular its commonest form, transitional cell carcinoma is a dichotomous disease which can present as a superficial lesion or an aggressive high grade lesion. There are strong links to environmental factors. The rising incidence and

high prevalence of transitional cell carcinoma mandate early and accurate diagnosis. The development of new techniques of management such as intravesical chemotherapy in tandem with the pathological diagnosis is improving the disease free interval and possibly rates of cure. However, it is well known that two morphologically similar tumours can behave differently, implying that the biology of transitional cell carcinoma remains poorly understood.

1.2 The Genetics of Cancer

Observations were made in the early 1900's linking cancer with cellular abnormalities. In his only theoretical paper, published in English in 1929, Theodore Boveri proposed that cancers arose from abnormalities that occur in chromosomes during faulty cell division. But it was not until the 1950's, when the technique of karyotyping was developed, that chromosomes could be identified, and another 20 years before the connection between abnormal chromosomes and cancer was made (reviewed by Bodmer & McKie, 1995).

Cancer has many causes. Biological, chemical and physical agents initiate or promote neoplastic processes. However, almost all carcinogens are genotoxic and cause somatic derangements (Cordon-Cardo & Sheinfeld, 1997). Several genetic aberrations are required for cells to develop full malignant potential (Nowell, 1976). Inherited (germline) and acquired (somatic) mutations can be implicated in this process. Genes involved in carcinogensis have been categorised as tumour suppressors or oncogenes. Recently a new type of carcinogenic mutation has been described, the mutator genotype (Loeb, 1997).

1.2.1 Genes involved in Carcinogenesis

1.2.1.1 Tumour Suppressor Genes

Tumour suppressor genes encode a diverse group of proteins which, through a variety of mechanisms, function to negatively regulate cell growth and development (Brown, 1997). Tumour suppressor genes have been recognised since the 1970's as a result of studying the patterns of inheritance of the childhood tumour retinoblastoma. Alfred G. Knudson (1978) in his "two hit" theory stated that genetic susceptibility to cancer could be explained by inactivation of one allele in the germ line of affected patients with the other allele then deleted or mutated in a somatic cell (reviewed by Brewster *et al*, 1992). This concept as applied to sporadic cancers involves a loss of function mutation of one allele followed by a somatic mutation affecting the other allele.

Genetic aberrations may occur by point mutation, rearrangement or deletion. Allelic loss, determined by loss of heterozygosity (LOH) analysis, is a hallmark of tumour suppressor loci. For example, the p16 protein plays a critical role in cell-cycle regulation via binding and inhibition of cyclin dependent kinases. The gene has been mapped to 9p21, a region which shows LOH in many carcinomas including those of

breast, lung, prostate and bladder (Foresman & Messing, 1997). The retinoblastoma (*Rb*) gene, also mutated in many cancers, encodes a nuclear phosphoprotein that functions as a cell cycle regulator (Romkes *et al*, 1996). Many tumour suppressor genes have key roles in cell cycle control, disruption of which can undermine genome stability.

1.2.1.2 Oncogenes

There are cellular genes (proto-oncogenes) that in another form (oncogenes) can cause neoplastic growth (Bishop, 1987). Specific alterations to these genes promote tumour growth in a dominant manner. A single "hit" may therefore activate these genes, usually by point mutation, amplification or gene rearrangement. Studies with retroviruses have shown that viral DNA can be integrated into host DNA, and the viral genes can then be expressed via the cell's own machinery (Bishop, 1987). Most proto-oncogenes, when expressed at high levels experimentally, can transform established cell lines. At least 40 oncogenes have been identified, with functions as varied as *c-myc*, which encodes a nuclear phosphoprotein involved in transcriptional regulation (Sauter *et al*, 1995c) and *c-erbB2* which encodes a transmembrane receptor-like protein with sequence homology to epithelial growth factor receptor (Coussens *et al,* 1985).

The action of tumour suppressor genes is in general opposite to that of oncogenes. Tumour suppressor genes are inactivated in cancer cells, and the introduction of functional tumour suppressor genes from normal cells into cancer cells may result in loss of tumourigenicity. In contrast, oncogenes are activated in cancer cells, and their introduction into normal cells leads to tumour formation (Sidransky, 1995).

1.2.1.3 Mutator Genes

A new class of recessive cancer genes (mutator genes) has recently been described. The expansion and contraction of tandem repetitive nucleotide sequences (microsatellites) in many human tumours, but not in non-malignant cells, provided the first strong evidence for a mutator phenotype in cancers. Tumours with microsatellite instability frequently contain mutations in mismatch repair genes (reviewed by Loeb, 1997). For example, the genes *MSH1* and *MLH1* encode proteins for DNA mismatch repair, loss of which would make cells replication-error prone, (reviewed in Reznikoff *et al*, 1996). Microsatellite instability has been investigated in many cancers although so far there has only been one heritable cancer syndrome causally linked to defective mismatch repair genes (including *MSH2* and *MLH1*), hereditary non-polyposis colorectal cancer (Lynch syndrome) (reviewed in Knowles, 1998). However, in sporadic

cancers such as bladder cancer there have also been reports of microsatellite instability (reviewed by Reznikoff *et al*, 1996).

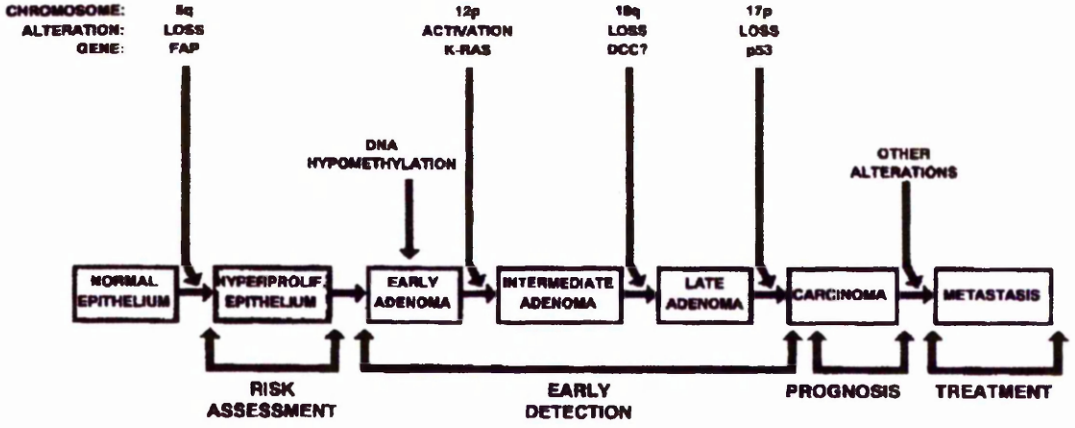
1.2.2 A Genetic Model for Cancer Progression

The model of tumour formation and progression proposed by Fearon and Vogelstein (1990) for colorectal cancer has become a paradigm for the study of many other cancer types (see Figure 5, Sidransky, 1995). This hypothesis proposes that progression from adenoma to carcinoma is based on specific genetic events that drive the carcinogenic process. For example, activation of the proto-oncogene *K-ras*, and inactivation of a TSG (*FAP*) on chromosome 5, occur early. Screening for these specific mutations, for example in stool specimens, in families with a history of colorectal cancer, could lead to early detection and treatment of pre-neoplastic lesions before they become malignant.

1.2.3 Genetic Models for Bladder Carcinogenesis

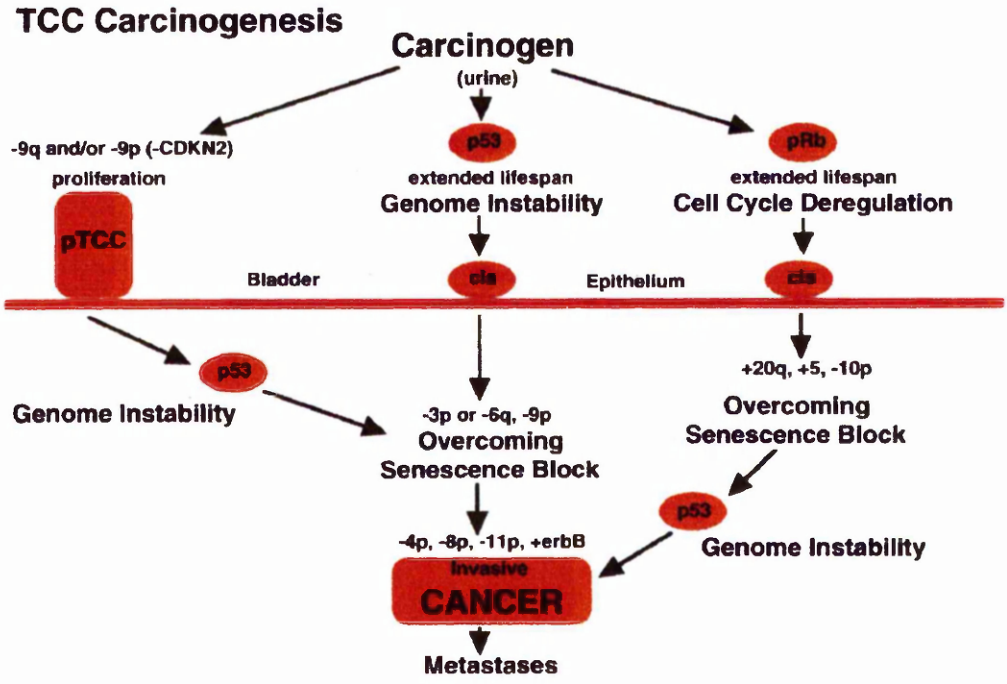
Transitional cell carcinoma of the bladder, as with many other solid tumours, is though to arise through the multistep accumulation of genetic aberrations (Saran *et al*, 1996). The model of urothelial tumourigenesis proposed by Reznikoff *et al*, (1996) illustrates this hypothesis (Figure 6). Reznikoff *et al*, (1996) suggest that at least three different genetic events that have been identified early in bladder cancer pathogenesis can be regarded as initiating events. This concept is partly based on research by Spruck *et al*, (1994), who demonstrated LOH of chromosome 9 in 34% of pTa lesions compared to 12% in carcinoma *in situ* (CIS) and flat dysplasia. In contrast, only 3% of pTa lesions contained a *p53* mutation while 65% of CIS and flat dysplastic lesions had such mutations. They suggested that the frequency of *p53* mutations could explain the propensity of flat dysplasia and CIS to progress, as *p53* mutations are known to destabilise the genome.

Figure 5: Genetic progression of colorectal cancer



The accumulation of genetic changes, not necessarily in the chronological order shown enables normal epithelium to progress to a highly malignant lesion

Figure 6: Model of different genetic pathways of urothelial tumourigenesis *in vivo*



This proposes pathways by which the variant forms of bladder cancer are initiated and progress to muscle invasion.

1.2.4 Molecular Genetics of Bladder Cancer

The commonest genetic and/or chromosomal aberrations are in *p53* (up to 70%), partial or complete loss of chromosome 9 (up to 60%), in *Rb* (30-40%) and loss of heterozygosity on chromosome 8 (approximately 23%). These are discussed in more detail below.

1.2.4.1 Chromosome 17

Currently the TSG *p53*, located at 17p13, represents the most frequently mutated gene in human cancers (Jones *et al*, 1991, Reznikoff *et al*, 1996). This crucial gene encodes a nuclear phosphoprotein, involved in cell cycle control and preservation of genomic integrity. It coordinates the cellular response to DNA damage and other cellular stresses by inducing cell cycle arrest (Lane, 1992). Deletion frequencies of *p53* can be as high as 70% in bladder tumours of high grade and/or stage. This gene has been widely studied (Spruck *et al*, 1994, Reznikoff *et al*, 1996, Popov *et al*, 1997), and mutations show a close correlation with progression to muscle-invasion and poor clinical outcome.

The gene *c-erbB2* is located at 17q21 (Coussens *et al*, 1985). Amplification and overexpression has been shown to correlate with poor clinical outcome in breast cancers (Özen, 1996). Correlation with outcome in bladder cancer has also been investigated. Gene amplification was seen only after progression had occurred and may be a potential diagnostic marker of progression (Underwood *et al*, 1995).

1.2.4.4 Chromosome 9

Partial or complete loss of chromosome 9 is a common early change with frequencies of up to 60% in all grades and stages of TCC (Knowles, 1995, Cordon-Cardo & Sheinfeld, 1997). In most low grade TCC's this is the only identified genetic aberration (Knowles, 1995). More extensive analysis has shown there are distinct deletion targets on 9p and 9q (Knowles, 1995). Four candidate TSG's have been mapped to chromosome 9; 9p21 is a common region of deletion shared by many carcinomas including bladder and this region contains the genes *p15*, *p16* and $p19^{ARF}$. High frequency homozygous deletion has implicated *p16,* also known as *CDKN2* or *MTS1* (Knowles, 1995) as the target tumour suppressor gene, but this remains controversial (Cairns *et al*, 1995, Dorkin *et al*, 1997). At least one candidate TSG has been proposed at 9q32-33, within the region 9q13-33 that contains at least two common

regions of deletion (Habuchi *et al* 1997). The proposed region at 9q32-33 has been designated *DBC1,* for deleted in bladder cancer gene 1 and a novel gene, *DBCCR1* has been identified (Habuchi *et al*, 1998).

An additional candidate region is at 9q34. The ABO gene locus has been mapped to 9q34.2, (Meldgaard *et al*, 1995). There is also a *TSC1* (for the congenital disorder tuberous sclerosis) candidate region at 9q34 (Wolfe *et al*, 1997). In addition, this region contains the oncogene *ABL* associated with the 9;22 translocation, which creates the Philadelphia chromosome in 95% of adults with chronic myelogenous leukaemia (Rowley, 1973). In conclusion, the high frequency of partial or complete loss of chromosome 9 appears to be an early event in bladder carcinogenesis and in particular in the initiation of transitional cell carcinoma (Figure 6).

1.2.4.3 Chromosome 13

Loss of function of the tumour suppressor gene, *Rb*, located at 13q14 (Cavanee *et al*, 1983), is associated with the development of 30-40% of human bladder cancers, especially high grade cancers with poor clinical outcome. However loss can also occur early in bladder cancer pathogenesis (see Figure 6, Reznikoff *et al*, 1996), thus this gene may be a potential marker of recurrence or progression risk.

1.2.4.4 Chromosome 8

Approximately 23% of TCCs have deletion of the short arm of chromosome 8. Loss of heterozygosity (LOH) has been associated with the development of an invasive phenotype in bladder cancer (Knowles *et al*, 1993, Wagner *et al*, 1997). LOH of 8p has been related to several other cancers, including breast (Ambrosone *et al*, 1996), colorectal (Farrington *et al*, 1996) and prostate (Matas *et al*, 1997) and may play an important role in the progression of prostatic and colorectal carcinomas (Takle & Knowles, 1996).

Regions of deletion have been mapped to 8p23.2-22 and 8p21.3-8p11.22 (Eydmann & Knowles, 1997). A candidate TSG homologous to the extracellular domain of the PDGF (platelet derived growth factor) receptor gene has been isolated from the region 8p21.3-22 (Takle & Knowles, 1996) and the genes for n-acetyl transferase 1 and 2 assigned to 8p22 (Hickman *et al*, 1994). These enzymes detoxify compounds such as aromatic amines. Exposure to these chemicals increases the risk of bladder cancer. A more proximal region of deletion, at 8p12-11.2 has also been identified. The candidate TSGs *POLB* and *PPP2CB* have been mapped to this region. Mutations in *POLB*, a DNA repair gene, have been described in both colorectal and

prostatic cancers, but *POLB* and *PPP2CB* are thought unlikely to be tumour suppressor genes in bladder cancer. Single strand conformation polymorphism analysis and direct sequencing of bladder tumours and bladder cancer cell lines failed to show tumour specific sequence variants (Eydmann & Knowles, 1997).

Amplification of chromosome 8 at 8p12, postulated to contain an oncogene, has been associated with breast and ovarian cancers (Theillet *et al*, 1993). This region contains the *PLAT* gene, which encodes the tissue-type plasminogen activator. Amplification at 8p12 has also been observed in bladder cancer (Wagner *et al*, 1997).

In conclusion, partial or complete loss of 8p is associated with the progression of several carcinoma types; to a lesser extent amplification of 8p12 is also implicated in carcinogenesis. The chromosomal region 8p thus has potential as a marker of progression.

1.2.4.5 Other Chromosomal Abnormalities

Many other chromosomal abnormalities have been described in TCC. Common regions of deletion exist on 4p, 11p and 14q (Knowles *et al*, 1994); frequent LOH on chromosomes 3p and 18q (Knowles, 1995, Reznikoff *et al*, 1996), and loss of chromosome Y (Sauter *et al*, 1995a). Other observations include abnormalities of chromosomes 1, isochromosomes of 5p and trisomy 7 (Waldman *et al*, 1991, Kallioniemi *et al*, 1995), overrepresentation of 1q31, 1p22, 3q24, high level amplifications on 20q (reviewed in Reznikoff *et al*, 1996) and polysomy X (Sauter *et al*, 1995a). Other potential tumour suppressor genes involved in bladder cancer are in the region 4p16.3 (Bell *et al*, 1996), 5p13-12 (Bohm *et al*, 1997), 3p13-14.3 (Knowles, 1995).

1.2.5 Molecular Diagnostic Markers

Deletion of 9q was demonstrated in all grade pTa and almost all grade pT1 TCC, whilst deletions of 3p, 5q and 17p are generally absent in pTa/pT1 carcinomas but are seen in muscle invasion (Dalbagni *et al*, 1993, Saran *et al*, 1996). LOH of 8p has been associated with both high tumour grade and stage (Knowles *et al*, 1994). Gains at 8q24 (containing c-*myc*) were observed at a higher frequency from pT1 to pT2-4 bladder cancers than from pTa to pT1 tumours (Wagner *et al*, 1997).

Higher tumour grade is correlated with deletion of 3p and 17p (Dalbagni *et al*, 1993) and increased copy number of chromosome 7 (Waldman *et al*, 1991). The

abnormal expression of retinoblastoma gene product (Rb) (Wright *et al*, 1995), *p53* and the proliferative marker Ki67 (Popov *et al*, 1997) have been linked to factors such as shorter disease-free interval, higher grade and poorer outcome for patients diagnosed with transitional cell carcinoma.

The observation that many human cancers express the enzyme telomerase, normally found only in germ cells, has prompted research into this area of molecular biology. The telomeric repeats amplification protocol (TRAP) assay to confirm the presence of the enzyme has produced promising results. For example in voided urine samples, all grade 1, 92% of grade 2 and 83% of grade 3 tumours were positive for telomerase activity in one study (Landman *et al*, 1997), suggesting that this may show a higher degree of sensitivity and specificity than previously reported with for example urine cytology. Reintroduction of a normal chromosome 3 to the human renal cell carcinoma line, RCC23, can restore the programme of cellular senescence. The loss of indefinite growth potential was associated with the loss of telomerase activity. This suggests that genes on chromosome 3 can repress telomerase activity as loss of chromosome 3, frequently observed in renal cell carcinoma, may allow a cell to divide indefinitely (Landman *et al*, 1997).

1.2.6 Summary

As with many other cancers, development of bladder cancer involves an accumulation of genetic aberrations. As understanding of the genetics of bladder cancer increases, the potential for molecular markers is becoming apparent as an adjunct to the existing clinical and pathological diagnoses.

1.3. Chemical Carcinogens and Bladder Cancer

The first link with environmental chemical exposure and the development of cancer was recorded by Percival Pott who, in 1775, noted the high risk of scrotal cancer in chimney sweeps and its association with prolonged contact to soot (Hill & Tannock, 1987, Jones *et al*, 1991).

1.3.1 Industrial Exposure

Rehn in 1895 reported "an undue incidence of bladder tumours in a group of workers employed in the manufacture of fuchsine (magenta)" and concluded that aniline was the most suspicious of the substances used in this process. From this stemmed the term "aniline tumour of the bladder" (Case *et al*, 1954). Rehn first linked urothelial cancer to occupational exposure (Hanssen *et al*, 1985). Throughout the first half of this century several animal studies using dogs substantiated these links (Case *et al*, 1954). Industrial exposure to α napthylamine, β napthylamine and benzidine are now generally accepted as causative agents in bladder carcinogenesis (Case *et al*, 1954). Industries such as the manufacture of aniline dyes, rubber and gasworks have been associated with occupational bladder cancer but β napthylamine manufacture was abandoned by the British chemical industry in 1952, and synthesis of other known carcinogens is now severely limited (Risch *et al*, 1995).

1.3.2 Environmental Exposure

Rachel Carson highlighted awareness of the effect of environmental pollutants in 1962 with the publication of her book "Silent Spring", and indeed prompted governments in many countries to restrict the use of pesticides (Carey, 1995). Most cancers are now thought to arise as a result of interaction of genetic factors, such as genetic polymorphisms, with environmental carcinogens (Perera, 1997). Up to 80% of cancers have been attributed to environmental factors (Smith *et al*, 1995).

Another potentially important source of exposure to bladder carcinogens is from benzo(a)pyrene, β napthylamine and other carcinogens found in cigarette smoke. In developed countries 40-70% of bladder cancers in men are causally linked to tobacco exposure (Sørlie *et al*, 1998). *p53* mutations occur in many human cancers, and links between smoking, *p53* mutations and the development of bladder cancer have been

investigated (Jones *et al*, 1991, Vineis & Martone, 1996, Sørlie *et al*, 1998). A current paradigm is that chemical carcinogens cause DNA adducts with resultant specific *p53* mutations, usually transversions (Reznikoff *et al*, 1996). Experimental studies have shown that after activation to electrophilic metabolites, potential carcinogens such as aromatic amines and benzo(a)pyrene may bind covalently to DNA, preferentially at the C8 position of guanine. This binding specificity is consistent with a mutation mechanism affecting primarily G:C base pairs (Sørlie *et al*, 1998).

To confuse the picture further, it appears that formation of DNA adducts and *p53* mutations in response to chemical exposure is strongly modulated by polymorphisms in genes responsible for metabolising aromatic amines, some of which are expressed on bladder epithelium (Perera, 1997). This is further discussed in Section 1.3.3.

1.3.3 Drug Metabolising Enzymes and Cancer Susceptibility

The ability to metabolise and thus detoxify xenobiotic toxins is one of the primary strategies that higher animals have developed to protect themselves against environmental insult. A number of enzyme superfamilies, such as the cytochrome P450s (P450s) and the glutathione S-transferases (GSTs) together with the two n-acetyl transferases (NATs), are thought to have evolved as adaptive responses to xenobiotic toxins. Thus variation in the activity of these enzymes could alter an individual's susceptibility to developing a toxin-induced cancer. It is now well established that the expression of many of these enzymes is genetically polymorphic and many published studies have attempted to relate the presence or absence of a particular allele to disease susceptibility (reviewed by Smith *et al*, 1995).

Drug metabolism in humans occurs in two distinct phases, as illustrated in simplified form in Figure 7 (Smith *et al*, 1995). Phase I catalyses the oxidation, reduction and hydrolysis of functional groups on drug and chemical molecules (Grant, 1991). The P450s are involved in at least 95% of this activity (Nebert, 1996). Phase II enzymes such as the GSTs and the NATs then increase the water solubility of the product sufficiently to permit excretion of the xenobiotic. Differences in activities of the enzymes involved in Phase I and II metabolism will affect the way the chemical is processed. Any abnormality in a Phase I enzyme, which could result in metabolic activation, has the potential to increase susceptibility to DNA damage and mutation,

rather than detoxification, and will have a more marked effect than a Phase II enzyme further down the metabolic pathway.

However minor metabolites of a parent compound which are particularly toxic may have significant phenotypic consequences if a Phase II enzyme is affected. In this case, the Phase II enzyme may not efficiently facilitate excretion of the metabolic products of Phase I activity, as will be further discussed in Section 1.3.4.

Differences in allele frequencies of metabolic enzymes have been observed between ethnic populations, for example the CYPD2D6 PM genotype from the cytochrome P450 family is present in 5 to 7% of the Caucasian population but is relatively rare in Orientals (Smith *et al*, 1995). The NATs also show ethnic variation between slow and fast acetylator phenotypes. For example, most populations in Europe and North America have 40-70% slow acetylators but in Asian populations only 10–20% are slow acetylators (Meyer, 1994). Low levels of either NAT or GSTM1 activity may influence the formation of *p53* point mutations. A proportion of bladder cancer patients who were carriers of these mutations were either homozygously deficient for GSTM1 (60%) or homozygous for the slow acetylator genotype in NATs (65%), (Brockmöller *et al*, 1996).

1.3.4 Metabolic Activity

Metabolic activation of aromatic amines is crucial for their carcinogenic potential (Frederickson *et al*, 1994). Figure 8 (Meyer, 1994) illustrates the pathways to bioactivation of an aromatic amine. There are competing pathways to bioactivation (via cytochrome P450 A2) step 1 or deactivation (via hepatic NAT2)-see step 2. The rate of deactivation is partly determined by the activity of NAT2, influenced by genetic polymorphisms. Further down the pathway, if the metabolite has not been successfully deactivated by n-acetylation and excreted, one of two activation pathways will be initiated. Either N-hydroxylation via P4501 A2 (step 3) followed by N,O transacetylation by cytosolic NATs (step 4) will occur or, following step 1, the toxic metabolite will be further bioactivated by NAT1, again influenced by genetic polymorphisms, expressed by the bladder epithelium (step 5).

The end result is the arylnitrenium ion [aryl-NH^+], the so-called ultimate carcinogen or mutagen, which forms covalent adducts by binding to and reacting with DNA. These DNA adducts can increase the rate of somatic mutation which if it occurs

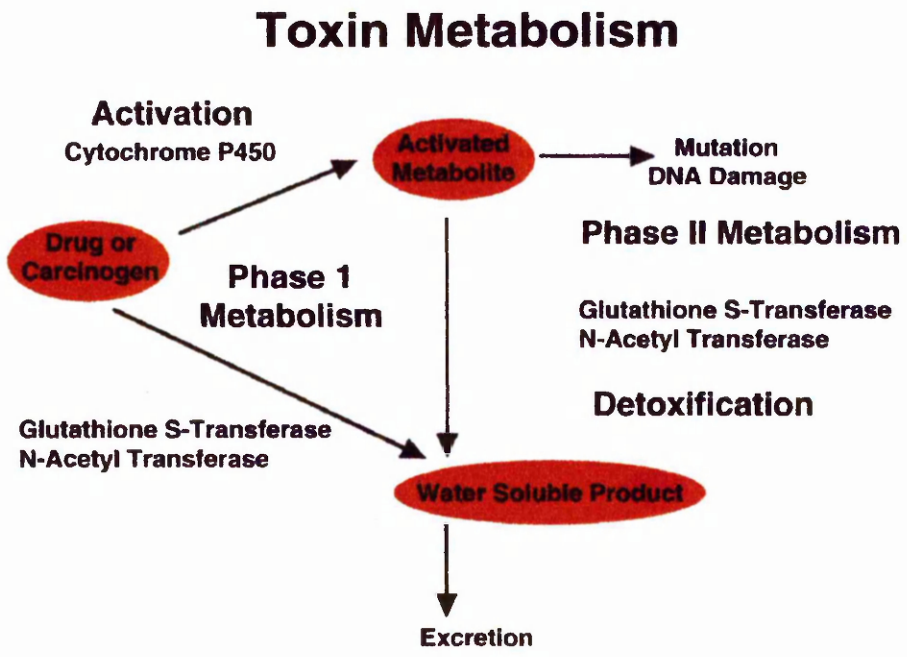
in genes controlling cellular proliferation can lead to a malignant phenotype (Grant 1993).

In addition, individuals with the slow acetylator phenotype who develop bladder carcinoma have higher circulating levels of 4 aminobiphenyl (ABP) haemoglobin DNA adducts, reflecting decreased clearance of reactive aromatic amine metabolites (Ambrosone *et al*, 1996). Vineis & Martone, (1996), reported that when investigating the relationship between ABP adducts and *NAT* polymorphisms, a combination of slow acetylator/fast oxidiser phenotype was associated with the highest level of ABP adduct. Studies measuring DNA adducts in workers exposed to benzidine, (DeMarini *et al*, 1997) are becoming more commonplace, and there may be a role for this type of assay to be used as a screen for cancer risk.

1.3.5 Summary

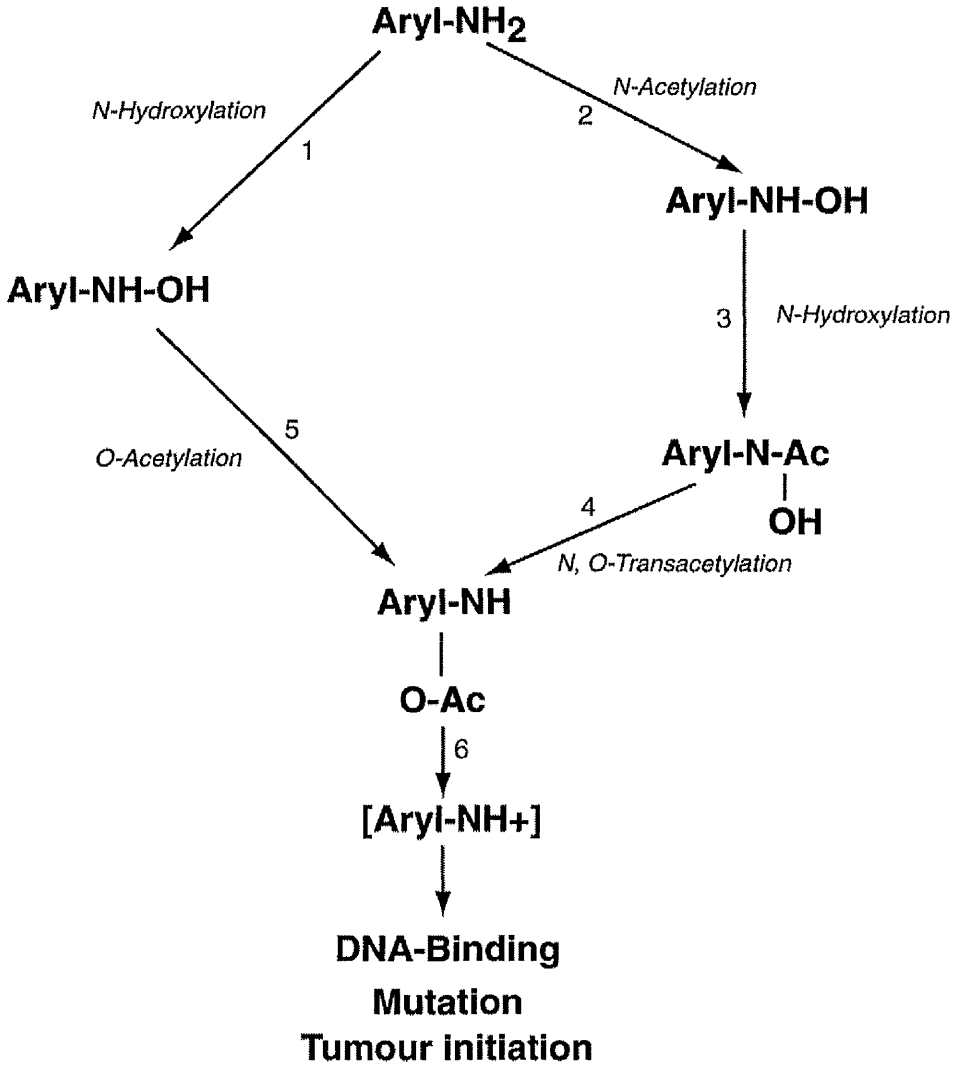
The development of bladder cancer has been linked with exposure to chemical carcinogens. The metabolic enzymes involved in detoxification or bioactivation, cytochrome P450s, NATs and GSTs, are genetically polymorphic and thus an alteration in their activity may result in a DNA binding mutation and subsequent increased cancer risk.

Figure 7: Pathways of drug metabolism



This diagram outlines the mechanisms whereby a potential carcinogen can be metabolised. Either activation or detoxification of the parent compound will occur, and the metabolic activities are dependent on phenotypic variations of the relevant enzymes.

Figure 8: Metabolism of Arylamines by Hydroxylation and Acetylation to Electrophilic Arylnitrenium Ions



This diagram illustrates the pathways involved in metabolic processes resulting in DNA damaging agents. This is a dynamic process dependent on the polymorphisms in the drug metabolising enzymes involved in these activities.

1.4 The N-acetyl Transferases

1.4.1 Phenotypic Analysis

Bönicke & Reif discovered the NAT acetylation polymorphism in 1953, following the advent of isoniazid therapy for the treatment of tuberculosis. The incidence of neurological side effects during therapy was related to elevated plasma concentrations of the unchanged drug. Impaired elimination was found to be related to a reduction in the rate at which isoniazid was enzymatically n-acetylated in the liver (reviewed by Grant, 1993).

From the early 1980's, the association with the acetylation polymorphism and individual susceptibility to the development of cancer was investigated. Compounds such as monoacetyldapsone/dapsone (Cartwright *et al*, 1982), sulphamethazine (Hickman & Sim, 1991) and caffeine (Kadlubar *et al*, 1992) were used to investigate phenotypic variation. Following oral ingestion of the drugs, the ratio of acetylated to non-acetylated metabolites was measured in urine (Hickman & Sim, 1991, Kadlubar *et al*, 1992), or plasma (Cartwright *et al*, 1982). These studies confirmed that inter-individual phenotypic variation existed. The drugs p-aminobenzoic acid and p-aminosalicylic acid were metabolised identically by slow and fast acetylators and termed "monomorphic" substrates, whereas isoniazid and procainamide were termed "polymorphic" substrates because the rate of acetylation correlated with distinct phenotypic variations. The existence of two different NAT enzymes was thus proposed.

Jenne (1965) suggested that the observed genetic variation was related to differences in the quantity of the enzymes rather than to altered kinetic characteristics of a structural variant and that the two different N-acetyltransferase enzymes were responsible for the metabolism of these distinct classes of arylamines (Grant, 1991, Meyer, 1994).

Experiments to elucidate biochemical and molecular mechanisms in humans showed that the majority of slow acetylators have less immunodetectable NAT2 protein in their liver cytosol relative to fast acetylators (Grant, 1993), supporting Jenne's hypothesis. The results of expression studies (reviewed by Grant, 1993) provided the first evidence that the human acetylation polymorphism was regulated at the *NAT2* locus, although it is now known that *NAT1* is also polymorphic (Bell *et al*, 1995).

The link between the slow acetylator NAT2 phenotype and bladder cancer is well documented (Cartwright *et al*, 1982, Grant, 1993, Risch *et al*, 1995, Badawi *et al*, 1995,

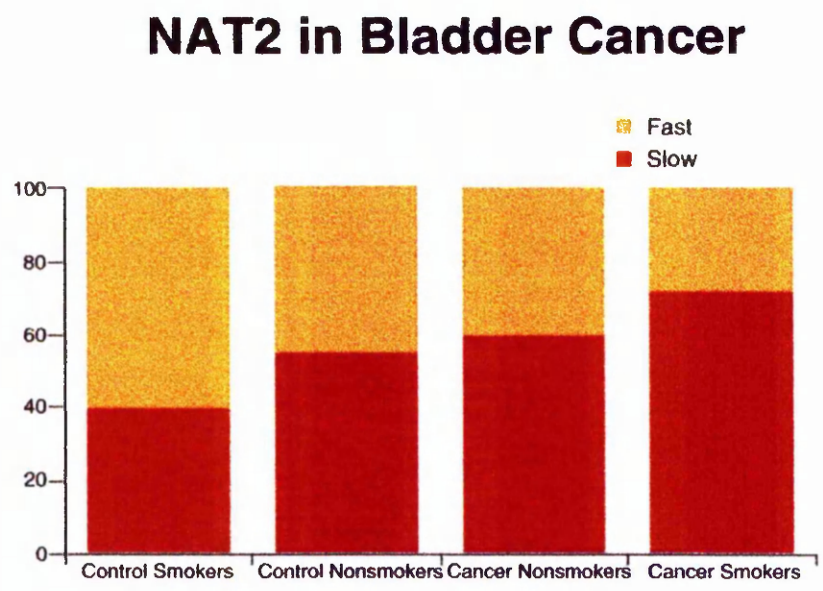
Ambrosone *et al*, 1996). Cartwright and co-workers, (1982), studied patients with bladder cancer and investigated their acetylation phenotype in response to previous research that suggested that the slow acetylator phenotype was a susceptibility factor in the development of bladder cancer. Twenty-three patients were dye workers and of these 22 had a slow acetylator phenotype, linking the metabolism of potential carcinogens with bladder cancer. This first strong association with acetylator phenotype and carcinogenesis was recapitulated by Risch and co-workers, (1995), using a similar cohort of patients to those in Cartwright's study. A greater proportion of patients with bladder cancer and previous occupational exposure to carcinogenic aromatic amines (71.0%) were slow-slow homozygotes for *NAT2* than in the control group (44.1%) (attending the urology clinic for non-malignant urological complaints with no previous exposure to carcinogenic aromatic amines). Interestingly, the proportion of bladder cancer patients with the slow-slow genotype without known previous occupational exposure to carcinogenic aromatic amines was also higher than the control group (65.4%).

In addition, the incidence of bladder cancer in smokers has shown a small but significant link with slow NAT2 acetylation phenotype (Golka *et al*, 1996, Okkels *et al*, 1997). In an analysis of the same patient cohorts, this time comparing smokers versus non-smokers in the control and bladder cancer patients, the proportion of slow acetylators of the patients with bladder cancer who smoked was 71.6% compared to 39.3% of smokers in the control group, as shown in Figure 9 (Risch *et al*, 1995). Slow acetylation phenotype has also been recently linked to the development of breast cancer (Ambrosone *et al*, 1996).

Less research has been carried out on an association with cancer risk and NAT1. However, NAT1 acetylates heterocyclic amines, associated with cooking of red meat and the consequent increased risk of colon cancer (Bell *et al*, 1995, Hubbard *et al*, 1998). NAT1 has also been linked with bladder carcinogenesis (Badawi *et al*, 1995, Bell *et al*, 1995).

In conclusion, interindividual phenotypic variation of the NAT's has been shown experimentally to be associated with differences in the rate of drug acetylation. Further research has substantiated the link between bladder carcinogenesis and the slow NAT2 phenotype. There also appears to be a relationship with colon carcinogenesis and NAT1.

Figure 9: Distribution of fast and slow NAT2 acetylator phenotypes amongst patient groups and controls, comparing smokers and non smokers.



A comparison of genotype with occupational exposure to chemical carcinogens, demonstrating a higher incidence of slow acetylators in bladder cancer patients that smoked (Risch *et al*, 1997).

1.4.2 Genetics

There are 3 distinct *NAT* genes; two loci encode the functional *NATs*, also known as *AAC1* and *AAC2*, characterised by their distinct but overlapping substrate specificities (Hickman *et al*, 1994). A third isolated region contains the related pseudogene *NATP*. Both the *AAC1* locus, also known as *NAT1*, and the *AAC2* locus, also known as *NAT2*, contain intronless genes that produce 290 amino acid proteins (Hein *et al* 1994). *NAT2* has been mapped centromeric to *NAT1* in the region 8p22 (Matas *et al*, 1997). This region is frequently deleted in bladder cancers of high stage and grade (Knowles *et al*, 1993).

1.4.2.1 Genotypic Analysis

Phenotypic analysis of an individual's drug metabolising capability cannot account for external factors such as smoking and alcohol consumption, and other factors such as impaired renal function may also affect drug clearance. It is now possible to genotype an individual and determine the relevance to disease susceptibility using DNA based techniques such as the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis (Smith *et al*, 1995).

1.4.2.2 *NAT1*

NAT1 was initially believed to be monomorphic (Smith *et al*, 1995) but recent studies have shown that it is indeed highly polymorphic and 9 allelic variants have been detected in humans (Hubbard *et al*, 1998). Polymorphisms in the polyadenylation signal have also been identified (Bell *et al*, 1995). Polyadenylation promotes RNA stability and it has been suggested that certain isoforms of *NAT1* (*NAT1*10*) are linked with a higher cancer risk because they are more stable resulting in an increase in NAT1 activity (Bell *et al*, 1995). *NAT1*10* contains a single base substitution in the polyadenylation signal in the 3' untranslated region of the gene. Bell *et al*, (1995) demonstrated that this allelotype conferred a 2.9-fold to 26-fold increase in the incidence of bladder cancers.

1.4.2.3 *NAT2*

Functional activity of *NAT2* is related to the inheritance of particular alleles (Matas *et al* 1997). Genetic variants have been cloned and sequenced. One major and two minor alleles confer the fast acetylation phenotype, other alleles are associated with slow acetylation at frequencies varying between different ethnic groups. Seventeen allelic variants have been identified in a Caucasian population (Agúndez *et al*, 1996).

Slow acetylation has been linked with cancer, particularly bladder (Cartwright *at al*, 1982, Risch *et al*, 1995,) and breast (Ambrosone *et al*, 1996). This may be due to LOH of 8p commonly seen in many cancers (and previously discussed in Section 1.2.4) which will confer a selective advantage for the slow acetylator genotype to become dominant. The impaired enzymatic function of the slow *NAT2* alleles gives a strong argument for the association with this allelotype and bladder carcinogenesis. Studies have shown there is a higher preponderance of slow acetylators with bladder cancer compared to disease free individuals (Cartwright *at al*, 1982, Risch *et al*, 1995).

1.4.3 Summary

Research into the phenotypic and genotypic properties of the NATs has increased our understanding of their metabolic activity, although the precise genetic mechanisms by which *NAT1* and *NAT2* influence carcinogenesis remain unclear. Their chromosomal location on 8p suggests they could be potential tumour suppressor genes either through LOH or loss of functional carcinogen metabolising enzymes.

1.5 Summary

The introductory chapter outlined the pathogenesis of transitional cell carcinoma of the bladder (TCC) and discussed difficulties of patient management which are exacerbated by the propensity of TCC to recur and to progress to muscle invasion. The molecular biology of cancer, and models for recurrence and progression in TCC were discussed and the reader's attention was drawn to the strong association between environmental exposure to potential chemical carcinogens and carcinogenesis, specifically, the aromatic amines with bladder carcinogenesis. The N-acetyltransferase genes, *NAT1* and *NAT2*, and their markedly polymorphic character, which may influence chemical carcinogenesis in the bladder, were discussed in detail.

The difficulty of identifying patients at greatest risk of recurring with bladder carcinoma makes additional tools for diagnosis and prognosis particularly desirable. The rapid development and constant refinement of molecular biology techniques has given researchers the opportunity to investigate the molecular mechanisms of carcinogenesis. Many genetic aberrations have been associated with cancer initiation and progression, with several genetic "hits" being necessary for cells to acquire the fully malignant phenotype. Against this background the time seems ripe to consider the rôle of molecular biology techniques as part of the clinical diagnosis and management of cancer patients.

The association between patient exposure to aromatic amines and bladder cancer gives us a clear "cause and effect" model within which to study carcinogenesis. Added impetus to the study of the genes *NAT1* and *NAT2* is given by their location to a region of frequent loss (on chromosome 8p) in many cancers including bladder cancer. The implied alteration of enzyme function in the aetiology of bladder cancer warrants further investigation.

1.6 Study Aims

The aims were to determine the chromosome 8 and *NAT2* status of patients presenting with transitional cell carcinoma, relating chromosomal aberrations to clinical course *i.e.* non recurrence, recurrence, recurrence and progression. This has the potential to increase the understanding of bladder carcinogenesis. For example: do patients with single episodes of non-detrusor-muscle invasive transitional cell carcinoma (TCC) have similar gene copy number to patients with a similar presentation but who subsequently develop detrusor-muscle-invasive TCC. To date this approach has been used by few researchers (Pycha *et al*, 1997, Bartlett *et al*, 1998).

There were two ways to address this question, using molecular biology, bearing in mind that the available material was formalin fixed and paraffin processed. Either by microdissecting tumour areas and using a method to investigate loss of heterozygosity such as restriction fragment length polymorphisms analysis (Hubbard *et al*, 1997), or using an *in situ* method such as fluorescent *in situ* hybridisation (FISH). An *in situ* method allows the tissue to be examined on a cell by cell basis, and avoids the problems encountered in trying to analyse homozygotes. FISH has been used extensively on many tumour types, including TCC, to determine gene and/or chromosomal copy number (Hopman *et al*, 1988, Sauter *et al*, 1995a, b, Wagner *et al*, 1997). Valuable data has been produced on the common genetic aberrations in TCC, but studies have tended to pick tumours from the archives or those presenting at clinic in a fixed period of time (*e.g.* Sauter *et al*, 1995c, Poddighe *et al*, 1996, Wagner *et al*, 1997). A larger amount of data would be obtained however if patients were followed sequentially, which could contribute to the elucidation of the mechanisms involved in recurrence and progression.

Thus the advantages of studying sequential tumours *in situ* from the same patient by FISH would allow the maximum amount of information to be gathered and therefore this method was chosen for the study. The study aims were therefore:

1. To develop a method to allow visualisation of chromosome 8 and *NAT2* in archival bladder tissue using the technique of fluoresecence *in situ* hybridisation.
2. To apply the method to normal bladder tissue controls and to bladder carcinomas.

CHAPTER 2: MATERIALS AND METHODS

2.1 Patient Data

2.1.1 Materials and Methods

Patient notes were accessed, and allocation to four groups was made, based on clinical history.

"Non recurrent" (NR) patients had one superficial or minimally invasive transitional cell carcinoma (TCC), (pTa/pT1) with no subsequent recurrences at check cystoscopy.

"Recurrent non progressive" (RNP) patients presented with several episodes of recurrent TCC following the index (primary) carcinoma but with no evidence of detrusor muscle invasion (stage pT2 and above).

"Recurrent progressive (RP) patients" presented with superficial or minimally invasive TCC and subsequent recurrences progressed to muscle invasion (pT2 and above).

"Progressed at presentation" (PP) patients presented with muscle invasive TCC (at least pT2).

Where available information on treatment (chemotherapy or radiotherapy), smoking habits and previous occupational exposure to carcinogens was recorded.

Patients whose index tumour or first recurrence was treated by diathermy without sampling the tumour for pathology were excluded.

2.1.2 Statistical Analysis

Differences in age at diagnosis were analysed by one-way ANOVA; length of follow-up and number of cystoscopies by Kruskal-Wallis tests (Minitab for Windows package, Release 9.2). For the sex ratio and deaths due to bladder cancer, Fisher's exact test (from SPSS for MS Windows, Release 6.1) was used.

2.1.3 Summary

Case notes were accessed, a patient cohort was identified, and all relevant details catalogued. Patients were categorised in four groups depending on the clinical course of their TCC.

2.2 Histology

2.2.1 Materials

Inclusion in the study was dependant on the availability of adequate tissue. This consisted of:

a) small biopsies of clinically "suspicious" areas (*e.g.* mucosal reddening) and random biopsies of other sites in the bladder
b) bladder tumour obtained from a transurethral resection (TURT)
c) cystectomy specimens.

2.2.2 Methods

The haematoxylin and eosin histology slides for each of the individuals identified were retrieved and re-examined microscopically with a pathologist within the Department of Pathology. Tissue sections were then recut from paraffin blocks containing tumour. Structurally normal bladder tissue was also retrieved to act as a reference control.

Ten consecutive 5μm sections were cut onto silanised slides. 3-aminopropyl triethoxysilane (silane, Sigma, UK) acts as a section adhesive, essential to prevent the tissue from becoming detached from the slide during the FISH assay. The method followed was:

Place slides into racks.

Place slide rack in acetone for 5 minutes.

Place slides in 2% silane (take 2ml of silane, add to 98 ml of acetone) for 5 minutes.

Wash under running tap water for 25 minutes.

Dry in fume hood.

Store in labelled and dated boxes.

The fifth section from each tissue block was stained with haematoxylin and eosin following the method outlined below:

Rehydrate sections through 2 changes of xylene and ascending grades of alcohol, from 100% to 70%.

Wash under running tap water.

Stain in haematoxylin (Gill's, Surgipath, UK)

Rinse in tap water.

Destain in 0.5% acid alcohol (99.5ml alcohol, 0.5ml 10N hydrochloric acid (Bancroft & Stevens, 1996)) for up to 20 seconds.

Rinse in tap water and "blue" in Scott's tap water substitute (2 g potassium bicarbonate, 20 g magnesium sulphate, distilled water 1 litre (Bancroft & Stevens, 1996)) for up to 1 minute.

Stain in 3% Eosin (3 g Eosin Y, 100 ml distilled water).

Rinse in tap water

Dehydrate through descending grades of alcohol, from 70% to 100%, then in 2 changes of xylene.

This protocol was performed on an automated system, the Stainette, E7751 (Miles Medical Laboratory Equipment Services, UK).

Care was taken to ensure there was as little wastage as possible between sections. Gloves were worn throughout and sections floated on a water bath containing distilled water. The haematoxylin and eosin sections of the bladder carcinomas were restaged and graded by the MRC reference pathologist for TCC, Dr K.M.Grigor (Edinburgh Royal Infirmary), using UICC guidelines (1978), whereby grade 3 and 4 are classified as grade 3.

2.2.3 Summary

Patients identified for the study were recruited if enough tumour material was available. Restaging and grading of the TCC's by a MRC reference pathologist enabled consistency of pathological reporting to be achieved.

2.3 Labelling of Probes

2.3.1 Probe Preparation and Labelling

Probes for FISH were initially prepared as crude isolates of nucleic acids with radioactive labels. With the description of nick translation (Rigby *et* al, 1977), the synthesis of biotin UTP and recombinant DNA technology (Warford & Lauder, 1991), probe preparation became simpler. The nick translation method developed by Rigby *et al*, (1977) utilised a simple technique that incorporated radioactive label following the introduction of "nicks" into DNA by the enzyme DNase I; 25 to 50% of the unlabelled nucleotides subsequently could be replaced by labelled nucleotides with DNA polymerase I via excision repair. Centromeric and telomeric probes for many of the

human chromosomes are available commercially, but for gene specific sequences probe generation is often carried out "in house". Biotin was the first non-radioactive label to be used and was incorporated into the nucleic acid in the form biotin 11 dUTP (Leitch *et al*, 1994). Other modified nucleotides are now available, and biotin 14 dUTP and biotin 16 dUTP are widely used. The label is incorporated at a position that does not interfere with hydrogen bonding between the probe and target DNA, containing a linker arm of at least 11 carbon atoms to minimise steric hindrance during hybridisation (Figure 10 Leitch *et al*, 1994).

2.3.2 Source of DNA

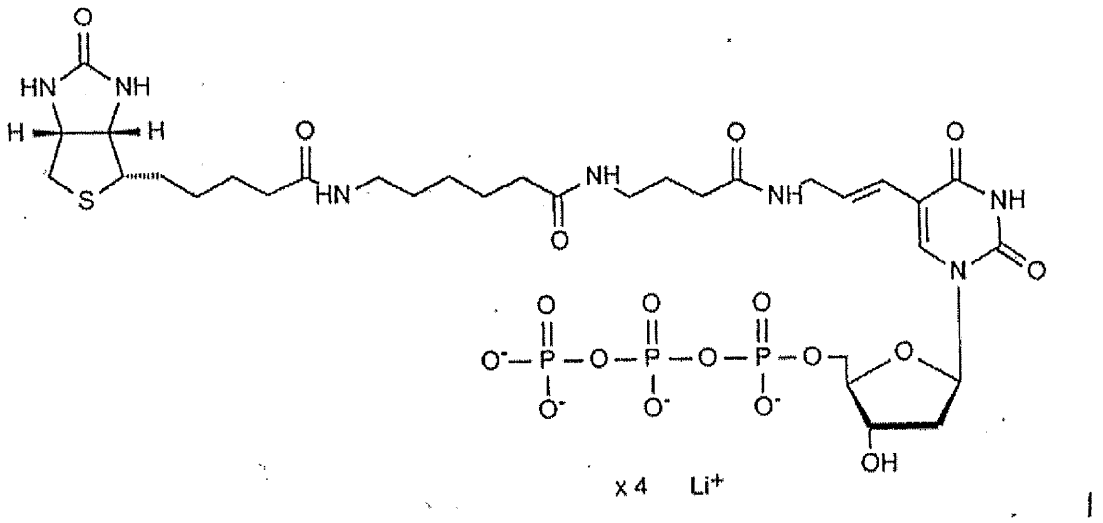
Cosmid DNA prepared from *Escherichia coli* transfected with *NAT2* cosmid (Franke *et al*, 1994) was kindly provided, together with the cosmid concentration, by the Department of Pharmacology, University of Oxford. The clones were tested by *NAT1* and *NAT2* specific PCR. Clones that were positive for both were rejected. DNA was received in TE (Tris-EDTA, Sambrook *et al*, 1989) buffer, pH 8.0.

2.3.3 Nick Translation

Enzymatic labelling kits are now available commercially and are used for *in situ* hybridisation (Hopman *et al*, 1991, Sauter *et al*, 1995a, b, c). The principles of the reaction are outlined as follows.

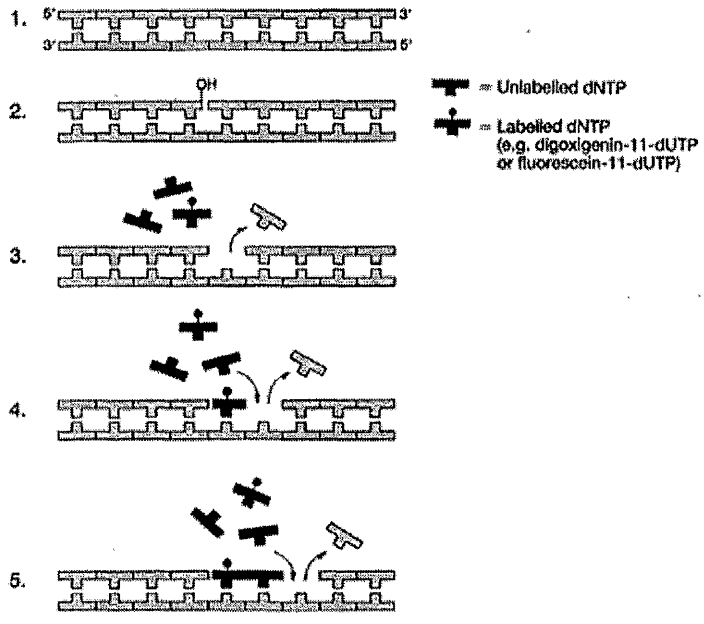
Two DNA enzymes, DNase I and *Escherichia coli* DNA polymerase I are used to incorporate labelled and unlabelled deoxynucleotide triphosphates (dNTP's) into double stranded DNA, (Figure 11). DNase I introduces single strand breaks or "nicks" in the double-stranded DNA to expose free 3′-OH groups (step 2, Figure 11). The 5′ to 3′ exonuclease activity of DNA polymerase I removes mononucleotides at the 5′ side of the "nick" (step 3, Figure 11). The DNA polymerase I then catalyses the incorporation of labelled and unlabelled dNTP's from solution at the 3′-OH end of the nick (step 4, Figure 11). The result is the synthesis of a new strand of uniformly labelled DNA, that includes labelled nucleotides (Leitch *et al*, 1994). The relative concentrations of DNase I and DNA polymerase I are altered to produce probes of between 200 to 600 base pairs which are most suitable for using in *in situ* hybridisation assays.

Figure 10 Biotin d-UTP



The atomic structure of biotin enables it to be incorporated into the relevant nucleic acid at a position that does not interfere with hydrogen bonding.

Figure 11: Nick translation



The principles of the reaction are outlined in diagrammatic form and further discussed in the text

2.3.3.1 The Nick Translation Method

The method employed was a modification of the Nick Translation System kit (Gibco BRL, UK) and is outlined as follows:

Combine the following:

1μg (10μl) DNA

5μl dNTP mix, final concentration is 0.02mM each dATP, dCTP,dGTP (Gibco BRL, UK);

10μl 5x biotin-16-dUTP (Boehringer Mannheim, UK), final concentration 0.004mM

6.6μl 1mM dTTP (Pharmacia Biotech, UK), final concentration 0.013 mM

12.4μl sterile distilled water.

Giving a total volume of 44μl

Microfuge.

Add 1μl DNase I (Boehringer Mannheim, UK), final concentration of 12μU/μl.

Add 5μl enzyme mix (DNA polymerase I, DNase I, Gibco BRL, UK), final concentrations of 0.08 U/μl and 8mU/μl respectively.

Place on a PCR block (Biometra UNO-Thermoblock, UK) pre-programmed to attain a temperature of 15°C for 90 minutes. Inactivate the enzymes by heating to 72°C for 10 minutes.

2.3.3.2 Confirmation of Product Size from the Nick Translation Reaction:

Take 8μl of product, add to 2μl of gel loading buffer, (Sambrook *et al* 1989). Gel loading buffer consists of 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in distilled water.

Take 1μl of 100 bp DNA ladder (Gibco BRL, UK), add to 2μl GLB.

Run on a 2% agarose gel. This concentration of agarose gel is recommended to resolve smaller DNA fragments (Sambrook *et* al, 1989) and is made up by adding 1g of agarose (Gibco, BRL) to 50ml 1 x Tris-acetate (TAE) buffer, from a stock of 50 x TAE buffer.

50 x TAE consists of: 242g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 Molal EDTA pH 8.0 dissolved in 1 litre of distilled water. Autoclave.

Heat the 2% agarose in a microwave to dissolve. Cool to approximately 50°C.

Add 2μl of 50mg/ml ethidium bromide (Sigma, UK).

Pour into a gel casting tray with a 12 lane comb and allow to set.

Place the gel in a flat bed electrophoresis gel rig (Hoeffer Scientific Instruments) with sufficient 1xTAE to cover the gel.

Run at 70V for 50-60 minutes.

View the size of the DNA fragment using an ultra violet light box. The product size is confirmed (it should be between 200 and 600 base pairs).

2.3.4 Suppression Hybridisation

Large DNA sequences such as a cosmid clone will often contain the sequences *Alu* and *Kpn* randomly dispersed approximately 900,000 and 100,000 times respectively, throughout the human genome (Gosden & Hanratty, 1991). Probes with *Alu* and *Kpn* sequences will hybridise to all chromosomes giving ambiguous results (Nisson *et al*, 1991). To avoid this, a product called human COT-1 DNA was developed, which is human DNA enriched for the major classes of repetitive sequences (Gosden & Hanratty, 1991). Unlabelled COT-1 DNA is combined with the labelled probe and the signal from repetitive sequences suppressed (Gosden & Hanratty 1991, Nisson *et al*, 1991). Hybridisation of the sequence of interest is then viewed against a low background of repetitive DNA sequences.

The addition of COT-1 DNA to nick translated DNA is a standard procedure to study genes by FISH either to establish loss or amplification. The presence of competitor DNA results in accurate hybridisation and increased sensitivity (Lichter *et al*, 1990). Therefore, the DNA probe labelled by nick translation was precipitated in the presence of COT-1 DNA as follows:

For every 5 µl (100 ng) of product, add 1 µl of 1 mg/ml COT-1 DNA (Gibco BRL, UK). Thus to 40 µl of product, add 8 µl of COT-1 DNA.

Add 1/10 volume 3 Molal sodium acetate pH 5.5 (Add 24.6 g to 100 ml distilled water. pH to 5.5) and 2 x volume cold 100% ethanol (stored at -20°C).

Precipitate the DNA at -20°C overnight or at -80°C for 20 minutes.

Microfuge for 15 minutes at 13000 rpm, decant the precipitant, wash in 80% ethanol, decant again and dry.

Reconstitute with 80 µl of hybridisation mix (from a stock of 5 ml formamide, 1 ml 20 x SSC, 0.5 ml 10 mg/ml salmon sperm DNA, 2 ml 50% dextran sulphate (All Sigma, UK) and 1.5 ml distilled water). Store at -20°C.

2.3.5 Summary

Nick translation was used to label the *NAT2* cosmid, to yield a probe of suitable length (200 to 600 base pairs) for *in situ* hybridisation assays. The labelled *NAT2* probe was then precipitated in the presence of COT-1 DNA, thereby minimising ambiguous hybridisation results.

2.4 Fluorescence *in situ* Hybridisation (FISH)

Double stranded DNA is held together by two hydrogen bonds between adenine/thymidine and three between cytosine/guanine base pairings. These bonds are relatively weak and it is possible to separate (denature) and reanneal (hybridise) strands of nucleic acid with relative ease. Therefore by attaching a label to a sequence of DNA, it is possible to hybridise this to a preparation containing a range of nucleic acids and specifically locate the target sequence containing the complementary base sequence (Figure 12, Herrington & McGee, 1992).

John and co-workers in the UK and Gall & Pardue in the US described the first *in situ* hybridisation (ISH) experiments almost simultaneously in 1969 (Warford, 1994). Radioactively labelled RNA probes were used to localise specific DNA sequences within the nuclei of *Xenopus laevis* (John *et al*, 1969). The resultant simplified technique of ISH/FISH has been widely applied to study chromosomal rearrangements, in chromosome mapping and has also been used to study numerical chromosomal and genetic aberrations in several tumour types. The technique is widely recognised as a powerful tool with which to study such genetic anomalies (Price, 1993, Waldman *et al*, 1991, Alcarez *et al*, 1994, Murphy *et al*, 1995, Nolte *et al*, 1996, Wolf *et al*, 1996, Bartlett *et al*, 1998 and many others).

2.4.1 Labels

Probes for FISH have been directly labelled with fluorochromes such as fluorescein isothoicyanate (FITC) and rhodamine (Leitch *et al*, 1994). Although this simplifies the technique, there is a resultant loss of sensitivity. This method is not recommended for the detection of low and single copy sequences (Leitch *et al*, 1994). However in cases of gene amplification, such as *cerbB2* in breast carcinoma (Pauletti *et al*, 1996), FISH with a directly labelled probe has been successfully utilised. The commonest non-radioactive labels are the haptens biotin or digoxigenin. Biotin has limitations as it is present as an endogenous component of many tissues (Warford & Lauder, 1991) and therefore non-specific binding of anti-biotin antibodies or streptavidin can be a problem.

Digoxigenin, a naturally occurring alkaloid derived from the foxglove (species *Digitalis purpura* and *D. lanata*) was utilised to circumvent the problems encountered with biotin. Both digoxigenin and biotin are frequently used in FISH as dual labels to

study the relationship between a unique sequence and the chromosome on which it resides (Han *et al*, 1994, Murphy *et al* 1995, Bell *et al*, 1996). This project used biotin as a label for *NAT2* as, in our hands, the biotin labelled probe was more reliable, producing a bright signal. Also, the antibody steps used to visualise the copy number following hybridisation with biotinylated probes have greater flexibility than for digoxigenin labelled probes. An alpha satellite digoxigenin labelled probe for chromosome 8 was used.

Figure 12 An Outline of *in situ* Hybridisation, Incorporating the DNA Labelling and Signal Detection Systems

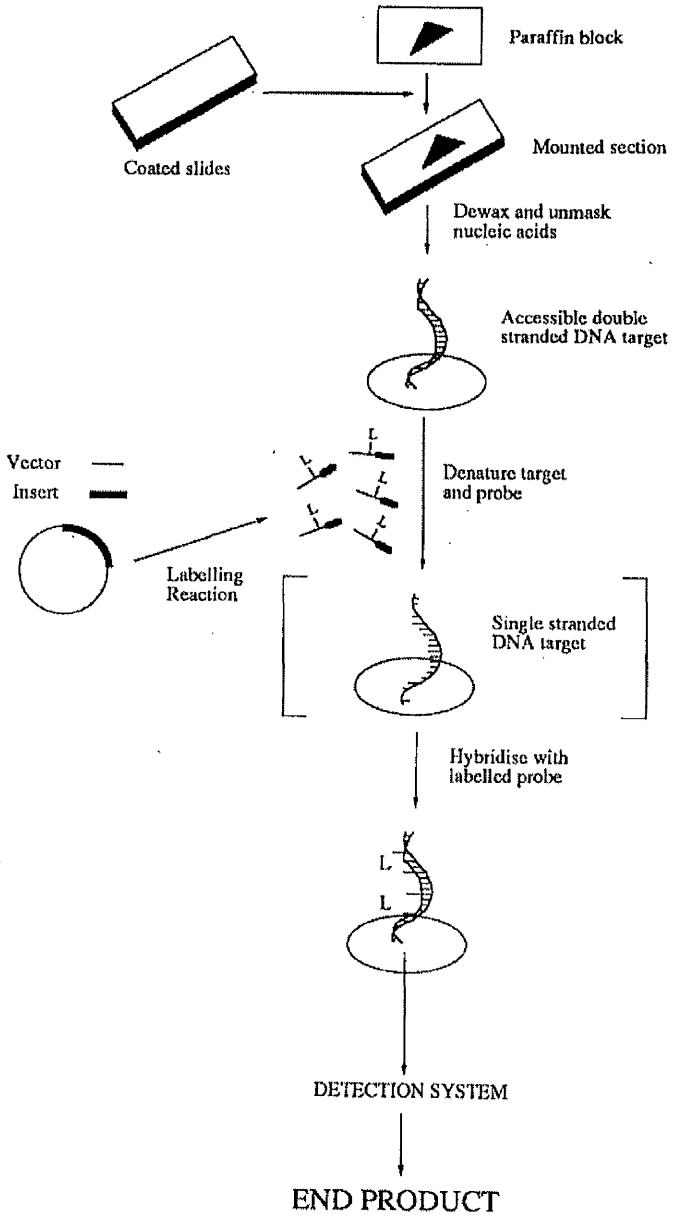


Figure 12: An outline of the principles of *in situ* hybridisation, further discussed in the text. Abbreviation: L= label;

2.4.2 Hybridisation

Hybridisation is carried out under low stringency conditions to enhance hybrid formation (Mitchell *et al*, 1992). The hybridisation mix contains reagents to allow the hybridisation to proceed as efficiently as possible. 2 x SSC and 50% formamide allow matched hybrids to form but because this is a relatively low-stringency mix, some mismatched hybrids will also form. However they will be less strongly bound than the correctly matched hybrids, and can be largely eliminated by the post hybridisation wash (see below).

Other constituents of the hybridisation mix are dextran sulphate, a macromolecule (molecular weight of greater than 8000) which increases the effective probe concentration by molecular exclusion (Herrington & McGee, 1992) and salmon sperm DNA which may reduce non-specific staining by blocking possible interactions with the phosphate groups of the nucleotide chain (Mitchell *et al*, 1992).

2.4.3 Detection of Hybridisation

Efficient high-resolution detection systems are a prerequisite for any *in situ* hybridisation study (Mitchell *et* al, 1992). Reagents labelled with FITC and Indocarbocyanine, Cy3 (Cy3) were chosen for use in this project. FITC is a widely used fluorochrome, as it produces a bright green fluorescence easily distinguishable from an appropriate nuclear counterstain. Ideally, a fluorochrome which fluoresces at the opposing end of the colour spectrum will allow clear differentiation between the probes.

A label that fluoresces red is usually used to contrast with FITC. Rhodamine or Texas Red were the first to be used but these are being superseded by the cyanine dyes, which include Cy3. These dyes are much brighter, more photostable and give less background than the traditional fluorochromes. A nuclear counterstain that fluoresces blue (4,6-diamidino-2 phenylindole-2 hydrochloride, DAPI) affords sufficient contrast from FITC and Cy3 and was used in this study.

2.4.4 Verifying Probe Specificity

The labelled probe was tested for specificity by co-hybridising to metaphase chromosomes with a probe for the centromeric region of chromosome 8. This was an essential preliminary step because the probe was then applied to interphase nuclei, which would give no indication of chromosomal specificity.

Dual fluorescence *in situ* hybridisation (FISH) with probes for the centromere of the relevant chromosome (chromosome 8) and for the gene of interest (*NAT2*) were applied to metaphase spreads of lymphocytes cultured from whole blood, prepared using conventional cytogenetic techniques, following a method based on Wheater & Roberts (1987). Cells previously screened for normal karyotype, kindly supplied by the Department of Medical Genetics, Yorkhill Hospital, Glasgow, were arrested in metaphase and fixed in 3:1 methanol:acetic acid. Slide preparation was carried out as follows:

Prepare the slides by soaking in 2% Decon 90 (Decon Laboratories Ltd., UK) overnight.

Rinse for several hours in running tap water.

Dry, place in 70% ethanol for 5 minutes, rinse in running tap water, store in the fridge for up to 2 days.

Make the metaphase spreads by centrifuging the cell suspension at 800 to 1000 x g for 10 minutes.

Remove the supernatant and resuspend in 3:1 methanol:acetic acid.

Take 20µl of the cell suspension and drop onto the top left hand corner of the slide. Allow it to run gently across the slide. Dry the slides and use the same day.

Confirm the presence of metaphases using phase contrast microscopy.

2.4.5 FISH

The principles of the technique are outlined in Figure 12 (Herrington & McGee, 1992). The method followed in this study is outlined below:

2.4.5.1 FISH for Chromosome Spreads: Preparation of Tissue for Hybridisation:

Denature cell suspension on the slide with 70% formamide 2 x SSC at 72°C for 5 minutes.

2 X SSC is a 1:10 dilution of 20 X SSC pH 7.0, consisting of 175.3g sodium chloride and 88.2g sodium citrate (both BDH, UK) added to 1 litre of distilled water, the pH verified and the solution autoclaved.

Dehydrate in ice cold 70% and 100% ethanol on ice and air dry.

Denature 10µl of biotin labelled *NAT2* probe per slide at 72°C for 5 minutes, allow to reanneal for 30 minutes at 37°C. This step is known as suppression hybridisation as discussed in Section 2.3.4.

Add 1µl of digoxigenin labelled chromosome 8 probe (Appligene Oncor, UK), denatured at 72°C for 5 minutes and stored on ice, to 10µl of *NAT2* probe. Cover with glass coverslips and seal with rubber cement (Halfords UK).

Hybridise for 16 hours at 37°C.

2.4.5.2 Post Hybridisation Wash

Peel off rubber cement.

Allow coverslips to fall off by immersing the slides in 2 x SSC for 15 minutes.

Place slides in 1 x SSC for 5 minutes at 72°C.

Rinse in PNT (phosphate buffer, pH 8.0 and 0.05% polyoxyethylenesorbitan (Tween 20)).

Phosphate buffer is 6.8ml 1Molal NaH_2PO_4, 93.2ml 1Molal Na_2HPO_4, (both BDH, UK), and 0.5ml Tween 20 (Sigma, U.K.) made up to 1 litre with distilled water, and the pH verified.

2.4.5.3 Histochemical Detection:

Block in PNTB ((PNT with 0.5% blocker) for 15 minutes.

The blocker is made up of 10% blocker (Boehringer UK) in maleic acid buffer, dissolved in the microwave The buffer consists of: 5.80g maleic acid (Sigma, UK), 4.38g NaCl, 3.50g NaOH (solid) (both BDH, UK) in 400 ml distilled water. pH to 7.5 and adjust final volume to 500ml.

Drain and cover with sheep anti-digoxigenin antibody (1µg/ml, Boehringer, UK) diluted in PNTB for 45 minutes.

Rinse in PNT for 10 minutes.

Rinse in 4 X SSCT (20ml 20 X SSC, 0.05ml Tween 20, 79.95ml distilled water)

Block in 4 X SSCTB (4 X SSCT with 0.5% blocker) for 10 minutes.

Drain and cover with FITC avidin (25µg/ml, Vectorlabs, UK) and rabbit anti-sheep (18µg/ml, Stratech, UK) diluted with 4 X SSCT for 40 minutes.

Rinse in 4 X SSCT for 10 minutes.

Drain and cover with Cy3 donkey anti-rabbit (3µg/ml, Stratech, UK) and biotinylated goat anti-avidin (5µg/ml, Vectorlabs, UK) diluted with 4 X SSCT for 30 minutes.

Rinse in 4 X SSCT for 10 minutes.

Drain and cover with FITC avidin (25µg/ml, Vectorlabs, UK) diluted with 4 X SSCT for 30 minutes.

Rinse in 4 X SSCT for 20 minutes.

Dehydrate through 70% and 100% ethanol, air dry and mount in Vectashield (Vectorlabs, UK) with 2μg/ml 4,6-diamidino-2 phenylindole-2 hydrochloride (DAPI) (Sigma, UK) added.

2.4.5.4 FISH for Formalin Fixed Wax Embedded Tissue Sections

Dewax and rehydrate sections:

xylene 2 x 10 minutes; methanol 2 x 5 minutes.

Air dry.

Treat with 30% sodium bisulphite (Sigma, UK) in 2 X SSC for 15 minutes at 45°C.

Rinse in 2 X SSC.

Treat with 0.25% proteinase K (Sigma, UK) in 2 X SSC for 40 minutes at 45°C.

Rinse in 2 X SSC.

Dehydrate through 70% and 100% ethanol, mount in Vectashield with DAPI and check digestion microscopically. Nuclei that stain grey to grey/blue are underdigested and, once the coverslip is removed, can be reintroduced to a fresh batch of proteinase K for up to 15 minutes. Nuclei that stain blue with clearly visible nuclear borders are suitably digested. Where nuclear borders are lost these sections are over digested and are discarded. The digestion is repeated with different sections for 30 minutes.

Rehydrate, treat with Streck ("Streck" Molecular Biology Fixative) (Alphalabs, UK)) for 10 minutes

Dehydrate through 70% and 100% ethanols.

Air dry.

Denature slides in 70% formamide 2 X SSC at 72°C for 5 minutes.

Dehydrate on ice in cold 70% and 100% ethanols.

Air dry.

Denature 10μl of *NAT2* probe per section at 72°C for 5 minutes and allow to reanneal for 30 minutes at 37°C.

Add 1 μl of chromosome 8 probe, (denatured at 72°C for 5 minutes and stored on ice) to 10μl (100ng) of the unique sequence probe for each section, cover with glass coverslips and seal with rubber cement

Hybridise for 16 hours at 37°C. Post hybridisation and immunocytochemical detection steps are carried out using the same method for chromosome spreads.

2.4.6 Visualisation

Stokes discovered the principle of fluorescence in the 19th century. He formed Stokes' Law that states that only short wavelengths of light can be used to excite fluorochromes, which then emit light energy of a longer wavelength. The emission of light energy enables the fluorochrome to be visualised. A fluorescence microscope is thus required, which is essentially a light microscope adapted by way of a specific illumination system, epi-illumination. Two types of filters are used to view the fluorescence: an exciter filter which selects short wavelength light and absorbs the radiation emitted by other wavelengths and a barrier filter, which allows only emitted light to pass to the viewer, absorbing the remainder. The filters need to be of high optical quality to enable the relatively small and faint areas of fluorescence to be viewed as clearly as possible against a dark background.

To view more than one label, incorporation of dual or triple band pass filters is required. In this study, a Leica DMLB microscope with a triple band pass filter system was used that allowed the combination of Cy3, FITC and DAPI to be visualised, excitation wavelength range, 420nm to 570nm; emission wavelength range 465nm to 640nm (Table 1). A 100 Watt mercury arc lamp supplied the short wavelength blue light. Viewing and scoring were performed with either a 40x Leica PL Fluotar oil immersion objective or a 100x Leica N PLAN oil immersion objective, and photomicrography performed using a Wild 48/52 photoautomat system (Leica, UK).

2.4.7 Control Sections

Morphologically normal bladder sections were cut for inclusion in the study to provide figures for normosomy. Initially 10 bladder sections were stained, and the results were the basis for normal values of chromosome 8 and *NAT2*. In all subsequent FISH experiments involving tumour slides, two normal bladder sections were also included, one following the FISH method (positive control), the other following the FISH method but excluding probe. At that stage, hybridisation buffer only was applied to the section (negative control). The positive control controlled for reproducibility and reliability of the technique, the negative control for non-specific staining generated by the hybridisation mix and antibodies.

2.4.8 Quantitation of Hybridisation Signals

To obtain the maximum data using the technique of FISH, a method of evaluating the results is required. Essentially evaluation involves counting the number of signals observed per interphase nucleus or metaphase spread.

The method for quantifying hybridisation signals generated from the centromeric probe was as follows: identify regions for analysis by FISH using adjacent haematoxylin and eosin stained slides and mark areas to be scored on an enlarged photocopy (200%) of the section (Figure 13). Analyse one to three tumour areas, depending on the section size. Count signals generated from each probe in 200 non-overlapping nuclei per area in the control and carcinoma sections using a multichannel counter. The number of signals (0 to ≥4) observed in each individual nucleus were recorded. Calculate mean copy number by dividing the total number of signals observed by total number of nuclei scored.

The mean copy number for chromosome 8 was then divided by the copy number for cosmid *NAT2* for each area assessed. This gave a measure of the relative hybridisation efficiencies of each probe. The technique was first applied to control sections (with normosomy for *NAT2* and 8) and a reference value was established. Subsequent analysis of tumour sections allowed comparison with this normal value and tumours with abnormal copy number were identified.

This study was carried out as a collaborative project with the Department of Pharmacology, University of Oxford, and two independent observers counted the signals.

2.4.9 Summary

FISH using biotinylated *NAT2* and digoxigenin labelled chromosome 8 was applied to chromosome spreads to verify probe specificity and then to tissue sections. Hybridisation was detected with anti biotin or anti digoxigenin reagents and visualised with fluorochrome labelled systems. Control sections were included to establish normosomy for both probes, and to check for hybridisation efficiency. Tissue sections were evaluated using a rigorous scoring system, a percentage by two independent observers.

Table 1 Fluorochromes Utilised

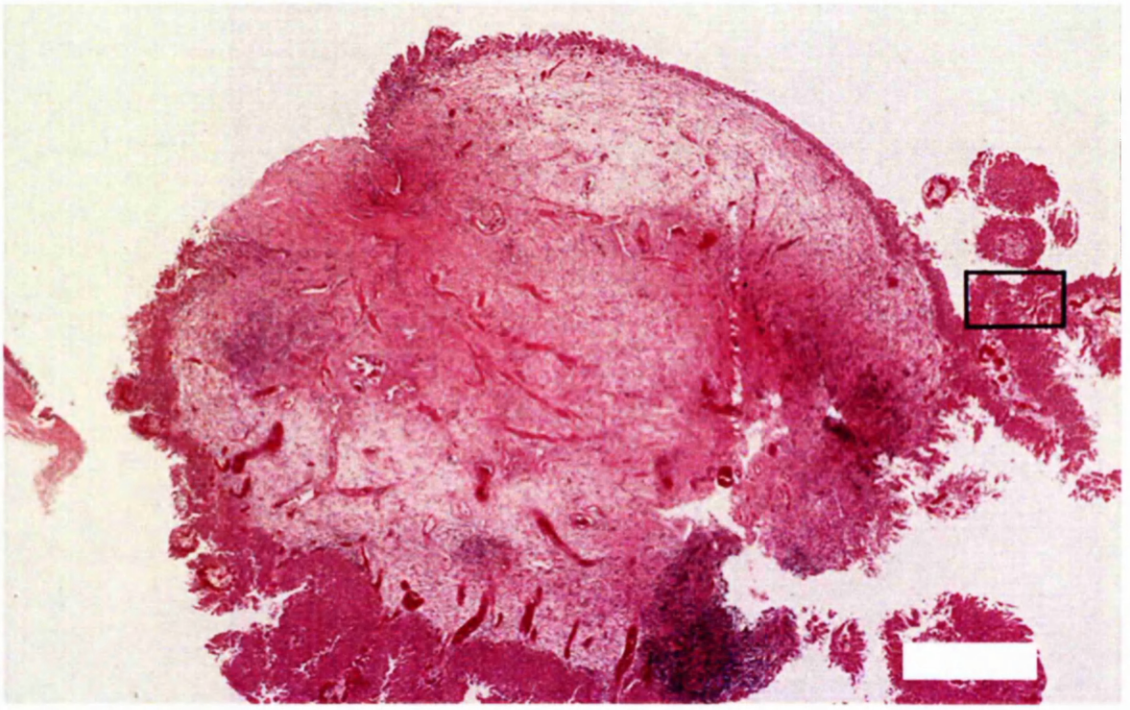
Fluorochrome	Excitation wavelength (nm)	Emission wavelength (nm)
Fluorescein isothiocyanate (FITC)	492	520
Indocarbocyanine, Cy3	550	570
4,6-diamidino-2 phenylindole-2 hydrochloride (DAPI)	355	450

The fluorochromes used in this study to visualise the *in situ* hybridisation are shown in this table, together with the wavelengths required to produce the appropriate signal

Figure 13: Haematoxylin & Eosin Stained TCC showing an area to be scored for FISH

Photomicrograph on the following page

This photomicrograph illustrates the identification of an area from an haematoxylin & eosin stained TCC, correlated with an adjacent FISH stained TCC to verify tumour areas for analysis; TCC pTaG2, magnification X 8, size bar=0.5mm.



CHAPTER 3 RESULTS

3.1 Patient Data

Information from the patients' notes was gathered to give a concise record of age at diagnosis, smoking and occupational history, the number of cystoscopies and other clinically relevant data. Nineteen patients were recruited into the study.

3.1.1 Data Derived from Patient Notes

3.1.1.1 Smoking and Occupational Histories

Twelve out of 13 (92%) patients, for whom the data was available, had a history of smoking. Six patients' smoking histories were not recorded in the case notes. Ten patients had occupational histories; only one had possible occupational exposure to carcinogens, a foreman painter (RNP1).

3.1.1.2 Patient Demographics

There were no significant differences in age at diagnosis, sex ratio, the number of cystoscopies and length of follow-up. Table 2 summarises data derived from patient notes with information about the primary carcinomas.

Patients presented with TCC within a similar age range, regardless of their subsequent disease course. Median follow-up was between 37 and 79 months, the longest being in the NR group. Five patients had follow-up of less than 36 months; two were lost to follow-up, one moved to another hospital and two died.

The NR patient group had the largest median number of cystoscopies, reflecting the longest median follow-up. Patients who presented with or developed detrusor-muscle-invasive disease (such as the RP and PP groups shown here) were followed up more frequently, compared to patients with non-muscle-invasive disease (such as the NR and RNP groups represented here).

Table 2 Patient Demographics

Patient group	NR (n=6)	RNP (n=4)	RP (n=4)	PP (n=5)
Age at Diagnosis	65.2 ±11.2 (51-78)	64.5±13.6 (51-82)	59.7±15.4 (43-80)	62.6±5.3 (56-70)
Sex M:F	6:0	3:1	3:1	2:3
Follow-up (months)	79.0 (24-106)	40.5 (18-135)	37.0 (14-152)	65.0 (24-151)
Number of Cystoscopies	11 (1-18)	7 (4-20)	7 (3-27)	8 (3-20)
Stage at diagnosis pTa pT1 pT2/2+	 5 1 0	 2 2 0	 2 2 0	 0 0 5
Grade at diagnosis 1 2 3	 3 2 1	 2 1 1	 1 2 1	 0 0 5

This table summarises the information gathered from patient notes

Abbreviations: NR = patients with non recurrent TCC

RNP = patients with recurrent TCC but which did not progress to detrusor-muscle invasion

RP = patients whose recurrences had progressed to detrusor-muscle invasion

PP = patients with detrusor-muscle invasion at presentation

n = number of patients per group

Age at diagnosis = mean in years ± SD. The range is in parentheses.

Follow-up = median in months. The range is in parentheses.

Number of cystoscopies = median. The range is in parentheses.

Regardless of clinical course, patients in the NR, RNP and RP groups had similar stage and grade at diagnosis. Stage or grade at diagnosis were not compared using any formal statistical analysis as the numbers were too small. The PP group was selected by muscle invasion (pT2 or above) at presentation.

3.1.1.3 Examples of Two Patients in the Study

Data obtained from one of the NR patients and one of the RP patients are shown in more detail (Table 3). Patient NR2 presented with a pTaG1 lesion, and was followed up until his death nine years later; no recurrences were detected during this time. An example of one of the RPs, patient RP1, presented with a pTaG1 lesion. After his first recurrence, a pT1G3 lesion, the patient was given palliative radiotherapy. He was followed up for 152 months (nearly 13 years), reflecting a long history of superficial carcinomas prior to muscle-invasion, and a pT2G3 lesion was reported at the last cystoscopy. Three months later he died due to metastatic bladder cancer.

3.1.1.4 Deaths Due to Bladder Cancer

Comparing deaths in the recurrer progressor (RPs) and progressed at presentation (PPs) patients, two of four (50%) of RPs and one of five (20%) of PPs died due to metastatic bladder carcinoma. Two of six (33%) of non recurrer (NRs) and two of four (50%) recurrer non progressor (RNPs) patients died of unrelated causes and one progressed at presentation patient died of unspecified causes. This suggested a trend but in such small numbers no conclusions could be drawn.

3.1.1.5 Chemotherapy and Other Adjuvant Therapies

None of the NRs was treated with chemotherapy; two of four (50%) of RNP patients were treated with epirubicin, one in addition with photodynamic laser therapy; 3 of 4 (75%) of RP patients had chemotherapy or radiotherapy; 2 of 5 (40%) of PP patients had radiotherapy, none had chemotherapy.

3.1.1.6 Summary

The information obtained from the case notes was collected and allowed the patients to be categorised depending on clinical outcome. This information was used to correlate the FISH experiments' results with genetic and chromosomal abnormalities and clinical events.

Table 3 Example of Two Patients in the Study

Patient group	NR	RP
Age at diagnosis	77	43
Sex	M	M
Follow-up (months)	106	152
Number of cystoscopies	11	27
Primary carcinoma	pTaG1	pTaG1
First recurrence		pT1G3
Preinvasion carcinoma		pTaG2
Postinvasion carcinoma		pT2G3

This table details clinical and pathological information from two patients within this study

Abbreviations: NR = non recurrer; RP = recurrer progressor

Primary carcinoma = stage and grade of primary carcinoma

First recurrence = stage and grade of first recurrent carcinoma

Last recurrence = stage and grade of last documented recurrent carcinoma

Preinvasion carcinoma = stage and grade of preinvasion carcinoma

Post invasion carcinoma = stage and grade of post invasion carcinoma

3.2 Pathology

Patient selection was followed by the identification of material from TCC specimens, stored in the pathology archives. Tissue blocks containing the maximum amount of representative tumour were selected for each TCC case in order that histology sections could be utilised to analyse tumour genetics by FISH.

3.2.1 Available Tumour Material

Three categories of available tumour material were included in the study:

3.2.1.1 Small Biopsies

For this investigation, a sub-group of patients were identified from a previous study, all of whom had sufficient material for analysis. Sometimes insufficient material remains within the paraffin block, as sections are routinely taken at several levels throughout a diagnostic biopsy thus ensuring that the possibility of missing tumour is minimised. In these cases, the remaining tumour biopsy material was inadequate for further study, Biopsies from all suspicious areas and any morphologically normal areas were included, (Figure 14B, pTaG2).

3.2.1.2 Bladder Tumour Obtained by TURT

Material from this category was not limited by sample size as two to six paraffin blocks were made from the resected tissue sent to the Department of Pathology. Tissue sections representing each block were examined microscopically, and those with the best preserved tumour included (Figure 14C, pTaG1). Some blocks contained predominantly muscle or inflammatory tissue and therefore were excluded.

3.2.1.3 Cystectomy Specimens

Inclusion of material from this category was again not limited by sample size, but by the amount of mucosa remaining on the cystectomy specimen. Cystectomy specimens received in the pathology department are usually opened to allow the fixative access to the tissues. The inner mucosal layers are susceptible to autolysis if this procedure is not carried out promptly and delays between operating theatre and laboratory are not unusual. All cystectomy tissue sections were examined, and those containing tumour with the best mucosal preservation selected for study. (Figure 14D, pT4G3).

Figure 14: H&E sections of Bladder Tissue

Photomicrographs are on the following page

14A: Morphologically Normal Bladder

This photomicrograph represents normal bladder mucosa, used in the analysis of normosomic values, magnification X 32, size bar = 0.2mm.

14B: Bladder Biopsy

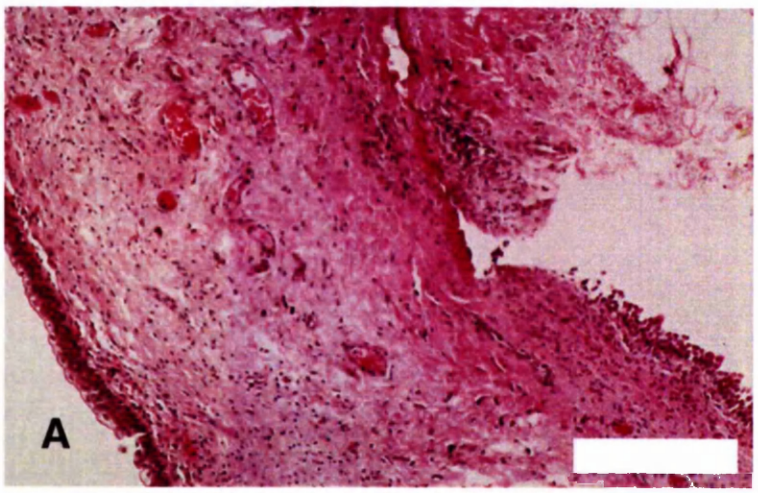
This photomicrograph represents a bladder biopsy, pTaG2; magnification X 25, size bar = 0.2mm.

14C: Transurethral Resection of Tumour

This photomicrograph represents a transurethral resection of tumour (TURT), pTaG1; magnification X 8, size bar = 0.5mm.

14D: Cystectomy

This photomicrograph represents a section from a cystectomy specimen, pT4G3; magnification X 8, size bar = 0.5mm.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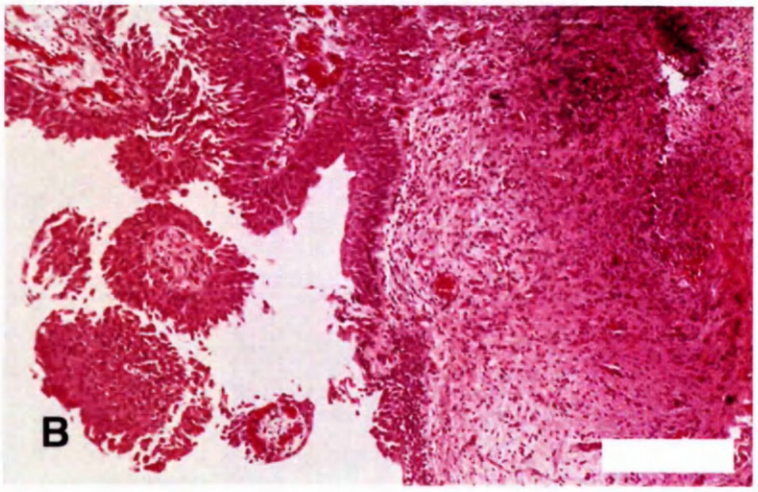
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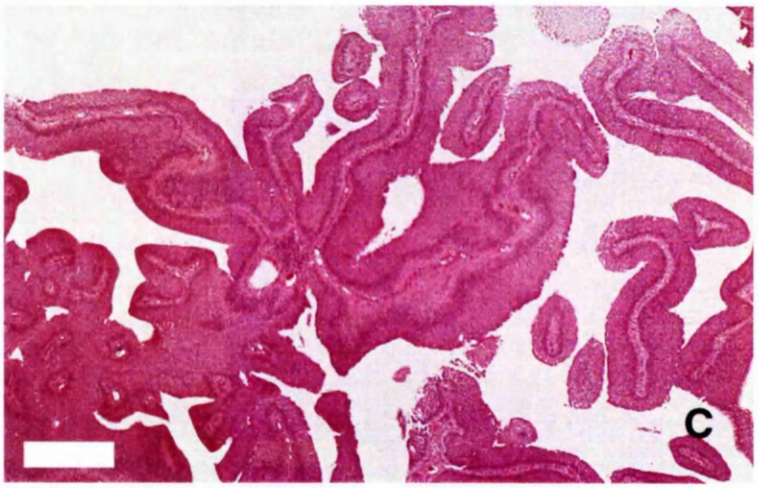
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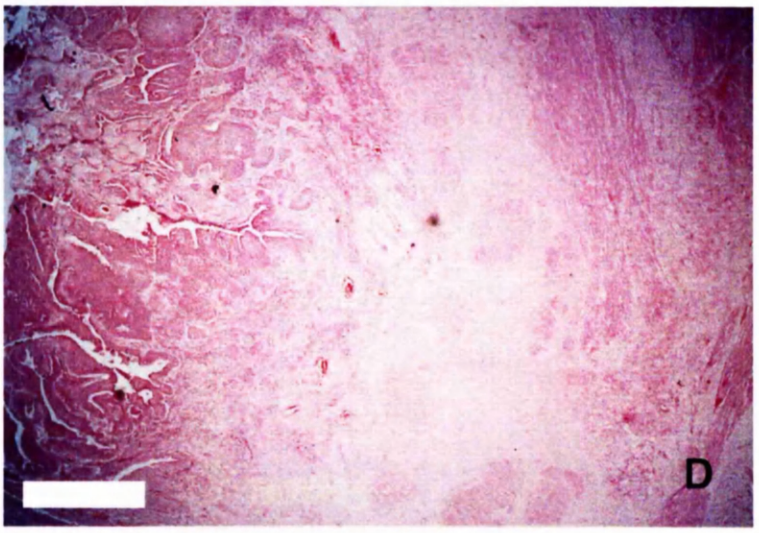
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3.2.2 TCCs Analysed

For NR and PP patients, the tumours analysed were: the primary (index) carcinoma, (n=11) and for RNP patients, the primary (index) carcinoma, first recurrence and last documented recurrence, (n=12). For RP patients, the primary (index) carcinoma, first recurrence, last pre- and first post-muscle-invasive carcinomas, were analysed (n=14). The total number of carcinomas analysed was 37. Photomicrographs of morphologically normal bladder mucosa and representative bladder carcinomas are illustrated in Figure 14, A to D.

3.2.3 Pathology Review

All TCC tissue sections were reviewed by Dr K.M. Grigor, Department of Pathology, Edinburgh Royal Infirmary. Three carcinomas were restaged from pTx to pTa and two from pTa to pT1 (as the base of the tumour was clearly seen at restaging): three were regraded from G1 to G2, and one from G2 to G3. In total, 24% (9/37) carcinomas were reported differently at review. Tissue sections were then prepared as outlined in Section 2.2.2.

3.2.4 Summary

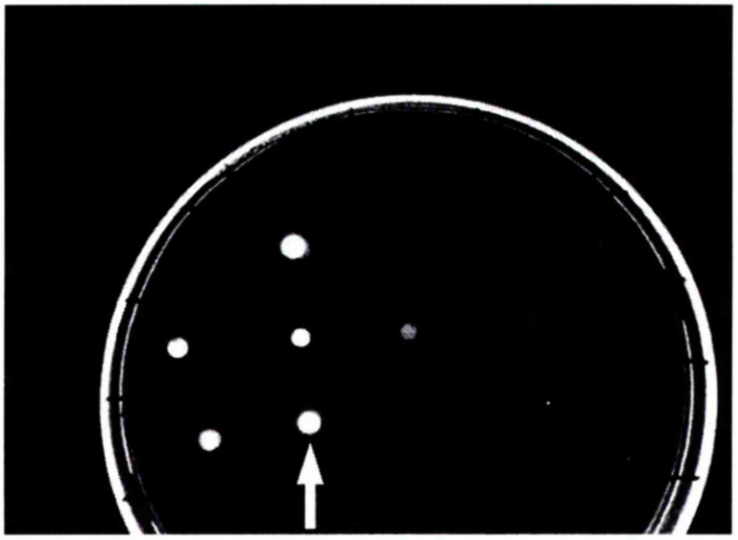
Tissue blocks for inclusion in the study were those most representative of the patients' carcinoma. These were sectioned and stored for use with the FISH protocol.

3.3 Nick Translation

3.3.1 DNA

The DNA for *NAT2* was accompanied by an image of the dot plate used to establish the concentration by comparing spots of known concentration with the unknown, (Figure 15, arrowed). 100ng/μl was received in 50μl of TE, pH8.0 and stored at -20°C. For nick translation, 1μg (10μl) of DNA was added to each reaction.

Figure 15: Dot plate



A range of concentrations of DNA were spotted onto a Petri dish containing an agarose gel. The intensity of the spot was then compared with an unknown spot to give a rough estimation of the DNA concentration. This was more reliable than OD values for DNA. The arrowed spot was the unknown, and the row above from left to right were concentrations of 100, 50, 25 and 12 ng/μl.

3.3.2 Investigation of the Nick Translation Method

3.3.2.1 Assessment of the Existing Nick Translation Protocol

The nick translation method using a kit (Gibco BRL) was performed, and the product run on a 1.5% agarose gel without pre-precipitation. The method is outlined as follows:

Combine the following:

10μl (1μg) DNA; 5μl dNTP mix: 10μl 5x biotin-16-dUTP; 20μl sterile distilled water, total volume 45μl. Microfuge. Add 5μl enzyme mix (DNA polymerase I, DNase I). Then proceed as outlined in sections 2.3.3.1 and 2.3.3.2.

The result was uncut cosmid, suggesting a lack of DNase I activity. This is shown in Figure 16A. Two samples of nick translated *NAT2* cosmid were run on the gel. Both show the characteristic intense bands at the top of the gel which represent high molecular weight cosmid. A 100 base pair ladder was run so that the fragment size could be estimated, and this is seen in the far right hand lane. The DNA ladder was run with each gel as an internal size marker. Bands of equal intensity were observed for 200 to 500 base pairs and higher intensity bands represented 100, 600 and 1 k base pairs.

On storage there is a progressive loss of DNase I activity after 4 to 6 months (Rigby *et al*, 1977). At this juncture the kit had been used for 5 and a half months. A new kit was ordered and the experiment was repeated. Similar results were observed impyling that the DNase I concentration was too low, rather than it being inactive.

3.3.2.2 Comparison of Two Nick Translation Methods

Two methods were thus compared, the nick translation protocol outlined in Section 3.3.2.1 and a non-kit protocol previously used to study *NAT2* copy number using FISH in bladder washings and exfoliated bladder cells (Stacey *et al*, 1996, 1999). The essential differences were that the former method used a dNTP mix of 0.2mM each dATP, dCTP and dGTP dTTP whereas the latter incorporated two-thirds the amount of dTTP and one third the amount of biotin dUTP. The existing method used 40mU/μl of DNase I whereas the alternative used 60mU/μl of DNase I.

The first method was specifically designed for radiolabelled nucleotides, but could be adapted for labelling biotinylated probes by incorporating a 1:2 ratio of biotin dUTP: dTTP. The stock solution of 0.4mM A, C, G, and T was further diluted to the correct proportions of 0.2mM A, C and G and 0.132mM T with additional 0.068mM biotin dUTP. This proportion of biotin dUTP:dTTP allows for the most efficient

incorporation of biotin into the DNA. At low concentrations, incorporation of biotinylated dUTP may be compromised by steric hindrance by the biotin molecule (Leitch *et al*, 1994).

The two methods were compared directly in one experiment. An identical amount of product (8µl, and 2µl of gel loading buffer) was run on a gel. The existing method showed a similar result to the gel picture in Figure 16A *i.e.* incut cosmid, whereas the product from the alternative could not be visualised (results not shown).

This implied that the enzyme had been too active in the latter method and had digested all the DNA. The conclusion was that a compromise between methods was needed, as there was not enough enzyme in existing method and excess in the alternative. It was now necessary to closely compare the concentration of enzyme required to produce fragments of optimal length. In order to simplify the results from these comparative experiments, control DNA (pBR322) was used and biotin dUTP omitted. The protocol outlined in section 3.4.2.1 was followed, the two variables to the original technique thus being DNase I concentration and nick translation reaction time.

3.3.2.3 Comparison of Nick Translation Times

The recommended time for nick translation is between 60 and 90 minutes (Leitch *et al*, 1994). However the alternative method stops the reaction at 90 minutes and if the fragments are not of correct size, the reaction is allowed to proceed for up to 20 minutes longer. Nick translation was carried out at 65, 90, 100, 110 minutes to establish whether the generation of fragments could be controlled by altering the time of the nick translation. The protocol outlined in section 3.3.2.1 was followed and the result was large fragments of equal size in each of the four lanes, 1 to 4, representing a reaction time of 65, 90, 100 and 110 minutes (see Figure 16B). The fifth lane in this gel contained the marker DNA. Lanes 6 to 9 contained duplicate results to lanes 1 to 4 results of the nick translation reaction followed as above, the only difference was that DNA from another cosmid was used to compare the results. However they were identical and the conclusion from this comparison was that not only did an increase of nick translation time have no effect on the size of fragments produced, but different sources of DNA also did not affect the reaction.

3.3.2.4 Comparison of DNase I Concentrations (1)

Several nick translation reactions were carried out in parallel using a range of DNase I concentrations. The alternative technique used 20mU/µl more than the kit (40mU/µl). Four dilutions, 40mU/µl, 50mU/µl, 62.5mU/µl, and 75mU/µl were

compared. Additional amounts of enzyme were added to the existing protocol as follows:

Take 1µl of stock DNase I, 10U/µl, dilute with 99µl sterile distilled water, giving a dilution of 0.1U/µl. Take 5µl of 0.1U/µl DNase I and add to 195µl sterile distilled water, giving a dilution of 0.0025 U/µl, or 2.5mU/µl.

Table 5 outlines the volume of DNase I and sterile distilled water added, to give a range of enzyme concentrations increasing by 10mU/µl. The enzyme was added, together with the existing enzyme mix after all the other constituents had been added. The reagents were placed on ice and added together also on ice. The result was uncut fragments with 40mU/µl lanes 1 and 2, and no evidence of DNA in the next 3 lanes, representing 50mU/µl, 62.5mU/µl and 75mU/µl respectively. (Figure 16C). The 100 base pair DNA ladder is in lane 6 to the right hand side of the fragments illustrated. In conclusion 40mU/µl of DNase I was insufficient to produce fragments of the correct size, whereas 50mU/µl of DNase I was an excessive amount.

Table 4 Comparison of DNase I Concentrations (1)

Final enzyme concentration (mU/µl)	40	50	62.5	75
Additional DNase I (µl)	0	4	9	14
Distilled water (µl)	27.5	23.5	18.5	14.5

The DNase I enzyme added was at a concentration of 2.5mU/µl.

3.3.2.5 Comparison of DNase I Concentrations (2)

The experiment was repeated with a range of DNase I concentrations from 40mU/µl to 49.6mU/µl with stepwise increases of 0.6 mU/µl, and two reaction times of 90 minutes and 110 minutes. Thus the protocol in section in 3.4.2.1 was followed with the addition of extra DNase I as follows. By taking 50µl of 2.5m U/µl and adding this to 150µl sterile water this gave a dilution of 0.6mU/µl. The volume of DNase I and sterile distilled water added to produce the stepwise dilutions is shown in Table 6.

The gel from this experiment is illustrated in Figure 16D. The far left hand lane (large arrow, lane 1), which incorporated a DNase I concentration of 42.4mU/µl produced fragments of optimal size, approximately 500 base pairs, after 90 minutes. When the reaction was allowed to proceed for another 20 minutes, fragments of

approximately 250 base pairs (small arrow, lane 2) were produced. Lane 3 incorporated a DNase I concentration of 44.8mU/μl following a reaction time of 90 minutes. This produced fragments of approximately the same size as lane 2. Lane 4 shows a far smaller fragment of approximately 100 base pairs when the reaction proceeded for a further 20 minutes. Lanes 5 and 6 incorporated a DNase I concentration of 49.6mU/μl and as before the reaction in lane 5 was stopped after 90 minutes, whereas that in lane 6 proceeded for a further 20 minutes (total of 110 minutes). Lane 5 shows DNA fragments of similar size to that in lanes 2 and 3 (250 base pairs), whereas lane 6 showed fragments of 50 base pairs. The results from the experiments that incorporated DNase I at concentrations of 40mU/μl to 41.2 mU/μl are not shown. The fragments produced by 40mU/μl showed uncut cosmid, a similar result to gel picture 16B; 40.6 mU/μl large fragments of over 1Kb; there was little DNA seen when 41.2 mU/μl of DNase I had been incorporated into the gel. These were the results following nick translation of 90 minutes.

When the reaction proceeded for a further 20 minutes, there was no change in the result for 40mU/μl, 40.6mU/μl produced fragments of about 600 base pairs and 41.2mU/μl produced a similar result to that of 90 minutes. The lack of DNA present in the gel from the reaction incorporating 41.2mU/μl may have been due to a pipetting error. In conclusion, the result shown for 42.4mU/μl after 90 minutes (lane 1, large arrow) was considered optimal for the production of suitably sized fragments for FISH.

Table 5 Comparison of DNase I Concentrations (2)

Final enzyme concentration (mU/μl)	40	40.6	41.2	42.4	44.8	49.6
Additional DNase I (μl)	0	1	2	4	8	16
Distilled water (μl)	27.5	26.5	25.5	23.5	19.5	11.5

The DNase I enzyme added was at a concentration of 0.6mU/μl.

3.3.2.6 Repetition of Experiment with Biotin dUTP & *NAT2*

As control DNA without biotin dUTP had been used to explore the parameters of time and DNase I, the experiments were repeated using control DNA and biotin 16 dUTP and dTTP in the recommended 1:2 ratio. Similar results to those in Figure 16D were obtained (results not shown). The experiment then incorporating the *NAT2* cosmid

instead of control DNA and the optimum concentration of DNase I of plus one either side. The reaction was carried out for 90 minutes. Figure 16E shows in lane 1 that incorporated 41.2mU/μl of DNase I which produced fragments of about 1500 base pairs, which was too large. Lane 2 (42.4mU/μl) produced fragments of about 600 base pairs, too large for *in situ* hybridisation experiments. This time, lane 3, (44.8mU/μl arrowed) produced DNA fragments of the correct size, of 400 base pairs. The 100 base pair DNA ladder is in lane 6.

The above modifications were incorporated into the nick translation procedure, and the results produced fragments of suitable length for use in FISH (Figure 16F). The probe was then used successfully in the FISH method as outlined in sections 2.4.4.1 and 2.4.4.2.

3.3.3 Conclusion

The most critical parameter in the nick translation reaction was the activity of DNase 1 (Leitch *et al*, 1994). Although a commercial kit ideally should perform under a wide variety of conditions, in this laboratory the enzyme concentration was insufficient and an additional 4.8 mU/μl of DNase I was required to produce fragments of the appropriate size (200-600 base pairs). Thus the modified protocol as outlined in section 2.3.3.1 was followed for the remainder of the project.

Figure 16: DNA Fragments Obtained after Nick Translation Experiments

Images are on the following page; a 100 base pair DNA ladder is included in each experiment.

16A: Assessment of Existing Nick Translation Protocol

In this gel image, two lanes of DNA, both of which show the intense bands characteristic of high molecular weight cosmid at the top of the gel. The far right hand lane contains the DNA ladder.

16B: Comparison of Nick Translation Times

In this gel image, large fragments of DNA of equal size in each of the four lanes, 1 to 4, representing a reaction time of 65, 90, 100 and 110 minutes. The fifth lane in this gel contained the marker DNA. Lanes 6 to 9 contained duplicate results to lanes 1 to 4 results of the nick translation reaction followed as above, the only difference was that DNA from another cosmid was used to compare the results.

16C: Comparison of DNase I Concentrations (1)

In this gel image, uncut fragments of DNA are seen in lanes 1 and 2, and no evidence of DNA in the next 3 lanes. The 100 base pair DNA ladder is in lane 6 to the right hand side of the fragments illustrated.

16D: Comparison of DNase I Concentrations (2)

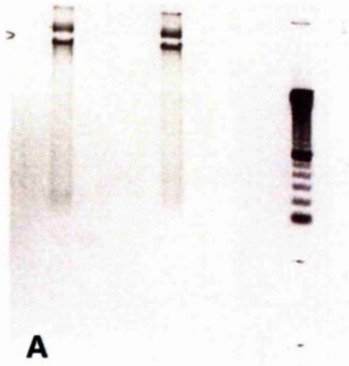
In this gel image, lane 1 (large arrow), illustrates fragments of optimal size, of approximately 500 base pairs; lane 2 illustrates fragments of approximately 250 base pairs and lane 3 illustrates fragments of approximately the same size as lane 2. Lane 4 shows a DNA fragment of approximately 100 base pairs. Lane 5 shows DNA fragments of similar size to that in lanes 2 and 3 (250 base pairs), whereas lane 6 showed fragments of 50 base pairs. The differences between the lanes of DNA fragments are explained in the text.

16E: Repetition of Experiment Including Biotin 16-dUTP and *NAT2* Cosmid

Lanes 1 to 3 show the different sizes of DNA fragments produced by altering the DNase I concentration, incorporating biotin 16-dUTP and *NAT2*. Lane 3, (arrowed) produced DNA fragments of the correct size, of 400 base pairs. The 100 base pair DNA ladder is in lane 6.

16F: Repetition of Experiment with Optimal DNase I Concentration

Recapitulation of the experiment shown in Figure 16E with the optimal DNase I concentration; DNA ladder on the right.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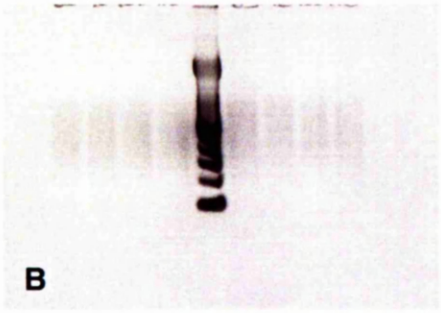
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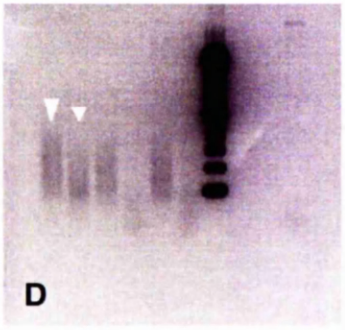
C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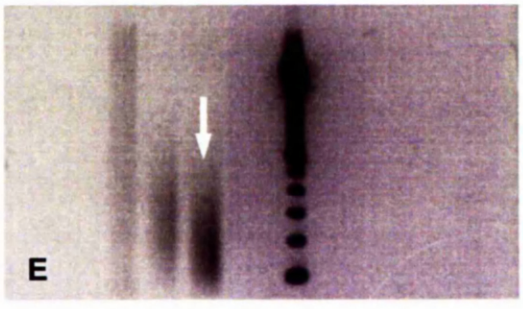
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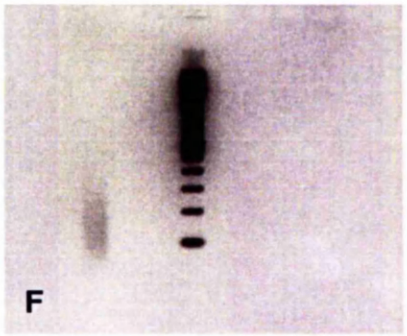

F

3.4 FISH: Modification for Formalin Fixed, Paraffin Processed Sections

The nick translated *NAT2* cosmid had previously been hybridised to exfoliated bladder cells from urine (Stacey *et al*, 1996), and bladder barbotage cells (washings) (Stacey *et al*, 1999) using the FISH technique. The cosmid was received in the laboratory and nick translated with a commercial kit. This was the first time the cosmid had been applied to formalin fixed paraffin processed material therefore extensive modifications of a previous technique were required. Morphologically normal skin sections were used principally in the evaluation and refinement of the methodology, together with chromosome spreads and bladder carcinoma sections where indicated.

3.4.1 Initial Evaluation

The method in place for commercial alpha or classical satellite probes was used for the first experiments. Formalin fixed, paraffin wax embedded bladder tissue sections were digested with 0.4% pepsin in 0.2 N HCl for 45 minutes; (chromosome spreads followed the protocol outlined in 2.4.4.1). 10µl (200ng) of biotinylated *NAT2* cosmid probe (supplied by M. Stacey) and alpha satellite chromosome 8 probe was applied per slide predenatured at 75°C for 5 minutes; the sections or spreads were denatured at 72°C for 4 minutes. Weak signal for both the alpha satellite (red) and *NAT2* (green) probes and precipitate were observed as shown in the photomicrograph, Figure 17A. Poor probe accessibility or purity and insufficient post hybridisation washes were suggested as possible causes of the weak signal and precipitate.

The DNA was precipitated as outlined in section 2.3.3.1 to increase purity, different digestion protocols were compared to improve accessibility of the probe as were various post hybridisation washes to eliminate precipitate.

3.4.2 Digestion

The material used in this study had been fixed with 10% neutral buffered formalin to preserve tissue morphology, therefore a tissue digestion step to unmask nucleic acids was required to perform *in situ* hybridisation. The standard fixative in histology laboratories, neutral buffered formalin, is an aldehyde that cross-links proteins and nucleic acids. Unmasking is achieved with limited proteolysis and must be optimised for each experimental system (Herrington & McGee, 1992).

Two digestion methods with paraffin embedded material and the *NAT2* cosmid probe only were compared. The DNA was nick translated following the method in section 2.3.3.1. DNA was precipitated before use as outlined in section 2.3.4. The existing method, previously used in this laboratory, had less steps at the digestion stage compared to the alternative (Table 6). The inclusion of 0.2N HCl and Triton X are tissue permeabilisation steps (Hopman *et al*, 1992). Normal skin and bladder carcinoma sections were used to make the comparison: using the existing method and skin sections, very weak patchy hybridisation with non specific staining was observed. With the alternative, nuclei were better preserved compared to the existing method, probably due to the shorter digestion time. More non specific staining in the alternative was observed compared to the existing method; hybridisation signal was comparable in both methods.

Using bladder carcinoma sections, no hybridisation and some non-specific staining was observed with the existing method. More precipitate was seen with the alternative method, largely obscuring assessment of nuclear preservation and possible signal, as shown in Figure 17, B and C. The conclusion was that the existing method gave better probe accessibility as less precipitate (non specific staining) was observed although the very weak signal suggested the accessibility could be improved.

An alternative digestion protocol, for unique sequence probes (Pauletti *et al*, 1996) includes a pretreatment step of 30% sodium metabisulphite followed by 0.25% proteinase K in 2 X SSC. Sodium metabisulphite is a strong reducing agent that destroys disulphide bridges in proteins (Ottaway & Apps, 1984). This step is particularly recommended with proteinase K and is thought to reduce autofluorescence and enhance the accessibility of target DNA (Lowery, 1998). Cosmid *NAT2* probe was applied with the alpha satellite probe for chromosome 8. A simple comparison between two bladder carcinoma sections was made, varying only the tissue digestion. All other methodological steps were identical. More green precipitate was observed with pepsin digestion; the nuclei stained a deeper blue (DAPI), indicating improved digestion and the *NAT2* signals (green) were more easily visualised with proteinase K digestion (Figure 18 A and B). In conclusion, the method for FISH was modified to incorporate sodium metabisulphite and proteinase K as the digestion steps. However the level of precipitate in the background was still high which led to an investigation of the probe purity.

Table 6 Comparison of Two Digestion Protocols

Existing method	Alternative method
0.4% pepsin in 0.2N HCl for 45 minutes at 37°C	0.2N HCl 10 minutes, PBS 2 x 5 minutes: 0.01% Triton X in PBS 3 minutes; PBS 2 x 5 minutes; 0.4% pepsin for 20minutes at 37°C

The components of two digestion methods used in the evaluation of the FISH methodology

Abbreviations: PBS=phosphate buffered saline

Figures 17-20: Evaluation of FISH Methodology

Photomicrographs are on the following four pages, red signal represents the chromosome 8 probe, green signal the *NAT2* probe, size bars 20μm and magnification X 320, unless otherwise stated.

17A: Normal Bladder using the Existing Protocol

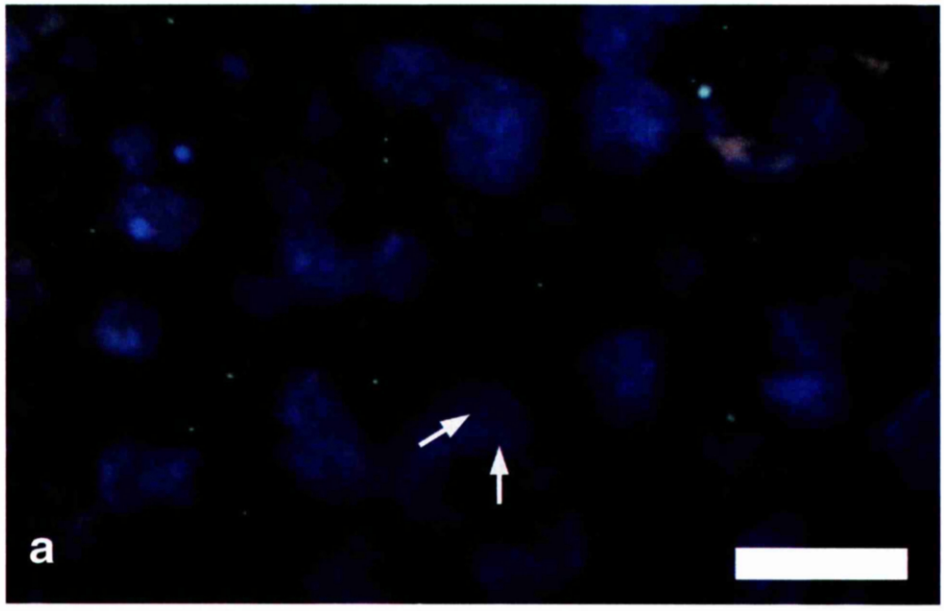
Preliminary experiment with the existing FISH protocol, arrows to the *NAT2* signal.

17B: Normal Bladder using the Existing Digestion Protocol

An exploration of the existing digestion protocol as a comparison with Figure 17C, magnification X 128.

17C: Normal Bladder using the Alternative Digestion Protocol

The alternative digestion protocol, showing excessive precipitate, magnification X 128.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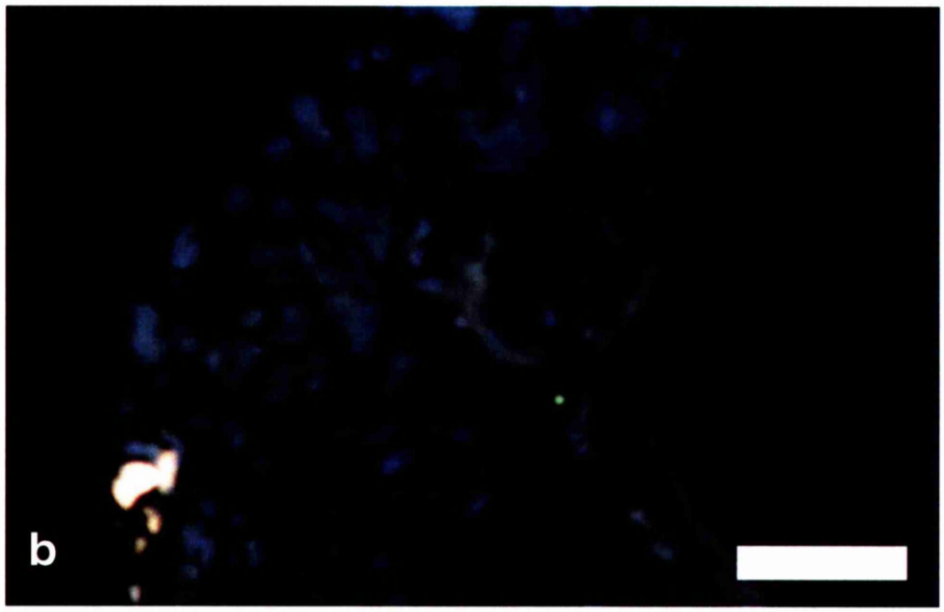
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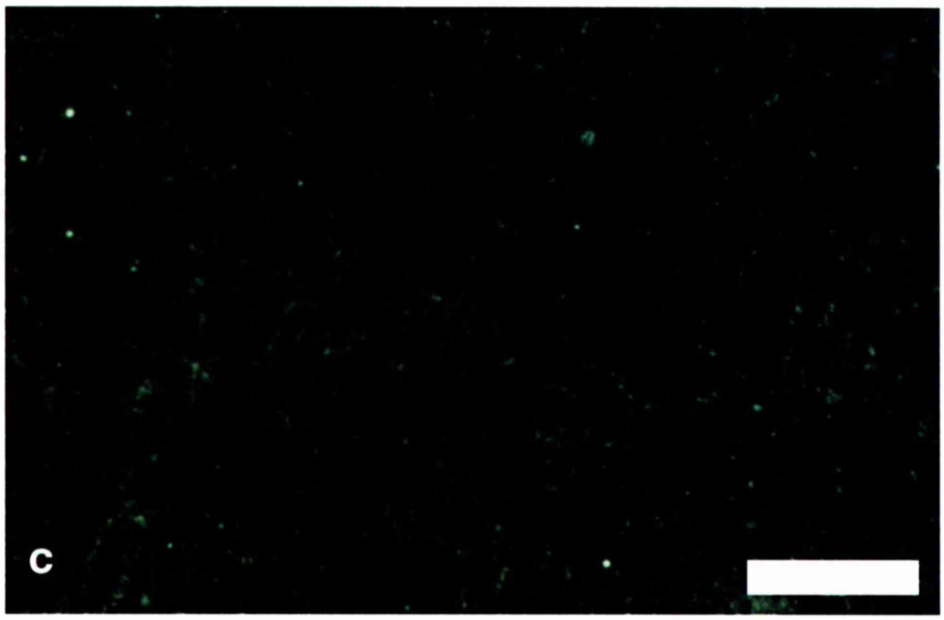

c

18A: Skin Tissue Section Digested with Pepsin & 18B: Skin Tissue Section Digested with Proteinase K

A comparison of two digestion methods using differing proteolytic enzymes, in both photomicrographs the *NAT2* hybridisation signal is arrowed.

18C: Normal Bladder Section Incorporating NICK Columns in the Technique & 18D: Normal Bladder Section Without NICK Columns in the Technique

A comparison of the FISH technique altering only the way in which the probe was purified, *NAT2* hybridisation signal is arrowed in Figure C.

a
c
b
d

3.4.3 NICK Columns

NICK columns (Pharmacia) are prepacked disposable columns containing Sephadex G-50 grade to separate nick translated DNA from unincorporated label. The aim of running the nick translation product through a NICK column, in this project, was to eliminate the precipitate seen at the post hybridisation stage, thought to be unincorporated label. The protocol outlined below was followed:

1. Allow the equilibration buffer (Pharmacia) to completely enter the gel bed. Add 100ml of 2.5% dextran sulphate and 2.5% eosin to the NICK column. Elute the solution followed by 10x 200µl 0.1 X SSC and 0.1% sodium dodecyl sulphate, SDS, (BDH, UK) and collect the fractions in a volume of 200µl. Note at which fraction the dextran sulphate (blue) and eosin (pink) are collected. Dextran sulphate has a high molecular weight similar to DNA; eosin a low molecular weight comparable with biotin. By noting at which fraction the two compounds are collected, this can be extrapolated to the nick translated DNA. Fraction 3 was blue and fraction 10 pink.
2. Allow the equilibration buffer (Pharmacia) to completely enter the gel bed. Add nick translated DNA to the NICK column. Elute the solution followed by 10x 200µl 0.1 X SSC and 0.1% SDS and collect the fractions. Fraction 3 was theoretically the DNA.
3. Precipitate DNA following the method in section 2.3.4.

DNA probe for *NAT2* that had been prepared using the NICK column was subsequently compared with the unpurified *NAT2* probe using the FISH method as outlined in section 3.3.1 and bladder sections. There was a distinct loss of signal following the use of the NICK column although the preparation was cleaner as demonstrated in bladder sections (Figure 18 C and D). The use of such columns is recommended for *in situ* hybridisation (Stickland, 1992), where less background was observed. However, the type of probe was not specified. A possible explanation for the lack of signal is that the biotin bound to the DNA was meshed in the Sephadex beads due to its shape. In conclusion, in these experiments, there was a lessening of hybridisation signal following the use of NICK columns. This step was therefore not incorporated into the methodology in this study based on the assessment of a single copy gene. This methodology may be beneficial in larger probes or possibly those labelled with digoxigenin.

3.4.4 Suppression Hybridisation

The addition of COT-1 DNA is recommended, when studying unique sequences (Lichter *et al,* 1990). The standard FISH method did not contain this step, and was thus compared with the addition of COT-1 DNA. Figure 19 illustrate the differences between the two conditions. Chromosome 8 is identified by the red hybridisation signal at the centromere. In the chromosome spread without the co-precipitation of COT-1 DNA (Figure 19A) weak cross reaction (red) with another two chromosomes was seen. No green signal (*NAT2*) was evident on the short arm of either chromosomes that had the red hybridisation signal. By contrast, the *NAT2* cosmid's signal was clearly seen in the chromosome spread on the p arm of chromosome 8 (Figure 19B, arrowed), when the cosmid DNA was co-precipitated with COT-1 DNA.

In the skin sections the hybridisation signal for chromosome 8 was clearly seen as two red dots in the nucleus. There was less precipitate when the cosmid DNA was co-precipitated with COT-1 DNA although the signal intensity was higher where the COT1-DNA had been excluded, but it was difficult to pick out the hybridisation signal (Figures 19, A and B). Some laboratories pre-precipitate the DNA with COT-1 DNA, (Murphy *et al*, 1995), others add it to the hybridisation mix (Bell *et al*, 1996 and Kallioniemi *et al*, 1995). In this study it was convenient to add the cosmid and COT-1 DNA preprecipitated to the hybridisation mix optimised for centromeric probes and thus the concentrations of either probe did not need to be altered. A recommended concentration of 10 times COT-1 DNA to cosmid DNA was used and found to be satisfactory (Lichter *et al,* 1990).

19A: Chromosome Spread Without Incorporation of COT-1 DNA & 19B: Chromosome Spread With Incorporation of COT-1 DNA

A comparison of the FISH methodology without and with COT-1 DNA to improve the *NAT2* probe purity, showing lack of *NAT2* signal in the chromosome spread without COT-1 DNA, *NAT2* arrowed in Figure 19B.

19C: Skin Tissue Section Without Incorporation of COT-1 DNA & 19D: Skin Tissue Section With Incorporation of COT-1 DNA

A comparison of the FISH methodology without and with COT-1 DNA to improve the *NAT2* probe purity, showing lack of *NAT2* signal in the skin tissue section without COT-1 DNA, *NAT2* arrowed in Figure 19D.

c
a
p
q

3.4.5 Post Hybridisation Wash

Probes are hybridised under conditions of low stringency, to allow complementary DNA strands to reanneal correctly. This also results in weakly bound mismatched hybrids that can be removed by a post hybridisation wash. This must be more stringent than the hybridisation conditions and is achieved by increasing the concentration of monovalent cation *i.e.* Na^+, increasing the concentration of formamide and/or increasing the washing temperature (Herrington & McGee, 1992). The 50% formamide 2 X SSC wash and 2 X SSC washes, both performed at 42°C for 20 minutes, used as the post hybridisation wash in the method for alpha and classical satellite probes, was directly compared to two other washes, a 2 X SSC or a 1 X SSC wash, both performed at 72°C for 5 minutes. Skin sections were used in this analysis. The protocol for precipitation with COT-1 DNA, tissue digestion and prehybridisation steps was followed as outlined in the Methods chapter, sections 2.3.4 and 2.4.4.2.

Photomicrographs for each of the posthybridisation washes are shown in Figure 20. The 50% formamide wash showed large green signals localised to the nucleus but also a high degree of green precipitate, but no red (chromosome 8) signal was evident (Figure 20A). The 1 X SSC and 2 X SSC washes both showed distinct signal for both the cosmid and centromeric probes (Figures 20 B and C, *NAT2* hybridisation signals arrowed). However, the cleanest preparation was seen with the 1 X SSC wash, with the least precipitate. High salt concentration solutions *e.g.* 2 X SSC are less stringent than low salt concentrations (such as 1 X SSC) (Mitchell *et al*, 1992) and are less likely to remove weakly bound non specific hybrids. For these particular probes, the higher stringency wash gave the most acceptable preparations, which allowed clear visualisation of the signal generated from the biotin labelled *NAT2* probe. The scoring results were also included to compare signal intensity following the 1 X SSC and 2 X SSC post hybridisation washes. As shown in Table 7 there was a higher percentage of nuclei with 1 or 2 signals in the section following the1 X SSC compared to the 2 X SSC wash. These data consolidated the choice of the 1 X SSC wash in the protocol.

20A: Skin Tissue Section With Existing Post Hybridisation Wash

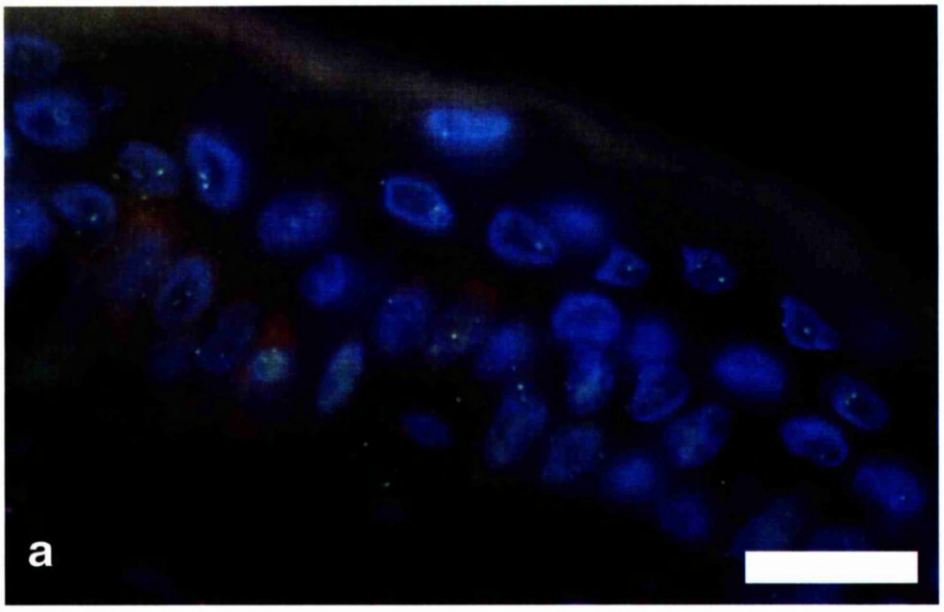
With the 50% formamide, 2 X SSC wash, excess green precipitate was seen, masking the *NAT2* signals

20B: Skin Tissue Section With 2 X SSC Wash

A lessening of precipitate was seen with this wash compared to Figure 20A, *NAT2* signal arrowed.

20C: Skin Tissue Section With 1 X SSC Wash

A lessening of precipitate was seen with this wash compared to Figure 20A, *NAT2* signal arrowed.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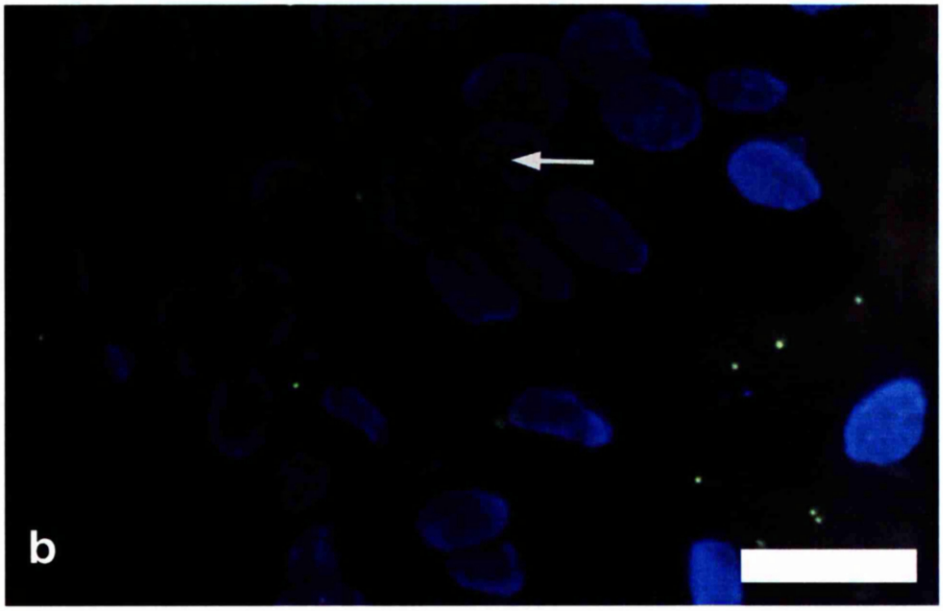
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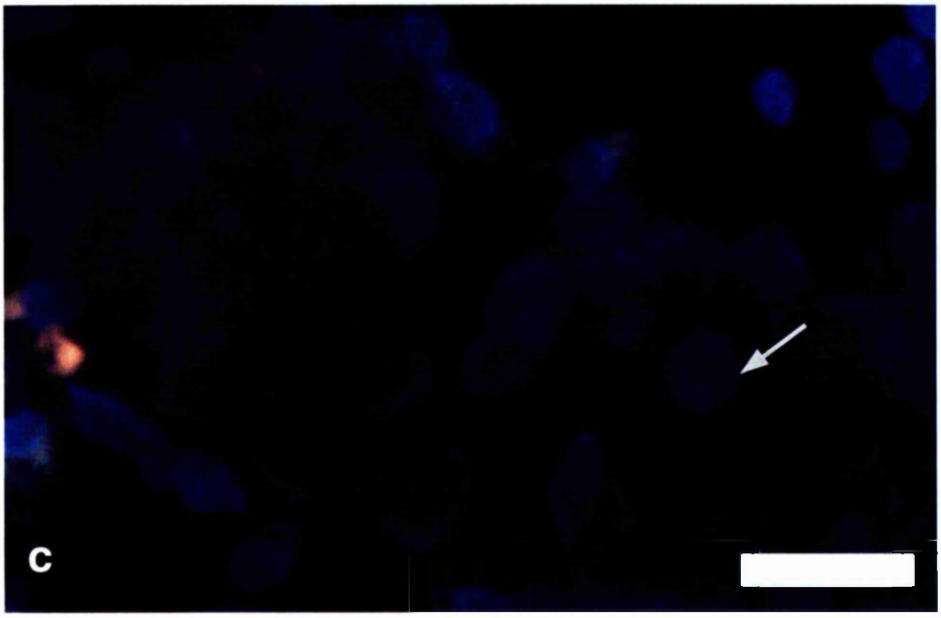

c

Table 7 Comparison of Scoring Results for Post Hybridisation Washes

PHW	Probe	0	1	2	Total	MCN	*NAT2*:8
2 X SSC	8	1	45	54	100	1.53	
2 X SSC	*NAT2*	42	39	19	100	0.77	50.33%
1 X SSC	8	3	31	66	100	1.63	
1 X SSC	*NAT2*	26	20	54	100	1.28	78.23%

This table shows the results of scoring two skin tissue sections varying only the post hybridisation wash; the scoring results show a higher number of hybridisation signals for both *NAT2* and chromosome 8 probes with the 1 X SSC wash.

Abbreviations: PHW = post hybridisation wash; 0, 1, 2 = number of signals per nucleus; MCN = mean copy number;

NAT2:8 = mean copy number of *NAT2* divided by chromosome 8

3.4.6 Summary of Modifications to the FISH Method

Various parameters of the FISH method were investigated, to attempt to overcome the presence of precipitate and to enhance the weak hybridisation signal. A competitior together with the *NAT2* cosmid probe reduced precipitate as did a proteolytic digestion step involving proteinase K as opposed to pepsin. A cleaner preparation was then observed with a 1 X SSC post hybridisation wash compared to any other wash. These modifications gave optimal results for the evaluation of the hybridisation signals and were adopted as the standard protocol.

3.5 Fluorescence *in situ* Hybridisation (FISH)

3.5.1 Metaphase Spreads

The cosmid had previously been used to demonstrate *NAT2* specificity (Stacey *et al*, 1996). The *NAT2* probe was hybridised to chromosomes arrested in metaphase together with a probe for the 8 centromere (Figure 21A) to identify chromosome 8. Hybridisation was seen on the p arm of chromosome 8 (arrowed) in the correct region for the *NAT2* gene. No other hybridisation signals were seen thus confirming probe specificity.

Figure 21: The FISH Protocol on a Chromosome Spread and a Range of Bladder TCC Sections,

The photomicrographs are shown in the following two pages, magnification X 320, size bars 20μm, unless otherwise stated.

21A: Chromosome Spread

This preparation shows *NAT2* probe specificity, to the short arm of chromosome 8, identified by the strong red hybridisation signal for chromosome 8; *NAT2* signals arrowed.

21B: Normosomy Chromosome 8 and *NAT2*

This TCC, pT1G2 is normosomic for chromosome 8 and *NAT2* (arrowed).

21C: Polysomy Chromosome 8, Normosomy *NAT2*

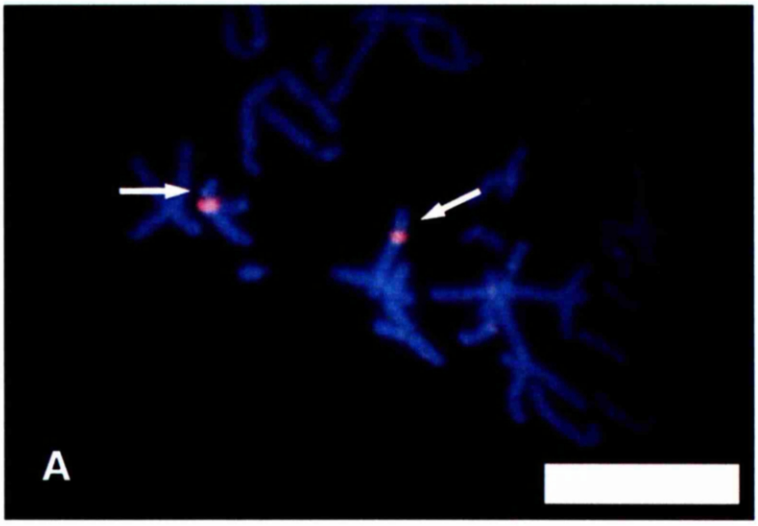
This TCC, pT1G3 is polysomic for chromosome 8, normosomic for *NAT2* (arrowed).

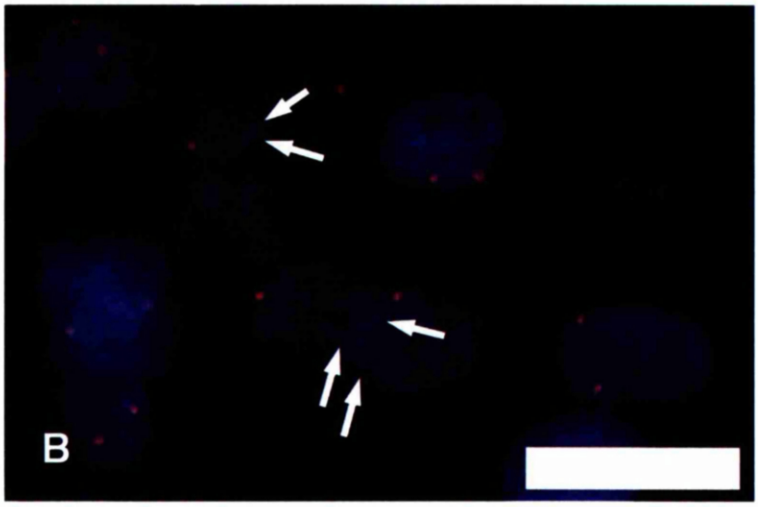
21D: Polysomy Chromosome 8 and *NAT2*

This TCC is polysomic for chromosome 8 and also shows multiple signals for *NAT2* (arrowed).

21E: Polysomy Chromosome 8 and *NAT2*

This TCC is polysomic for chromosome 8 and also shows multiple signals for *NAT2*.


A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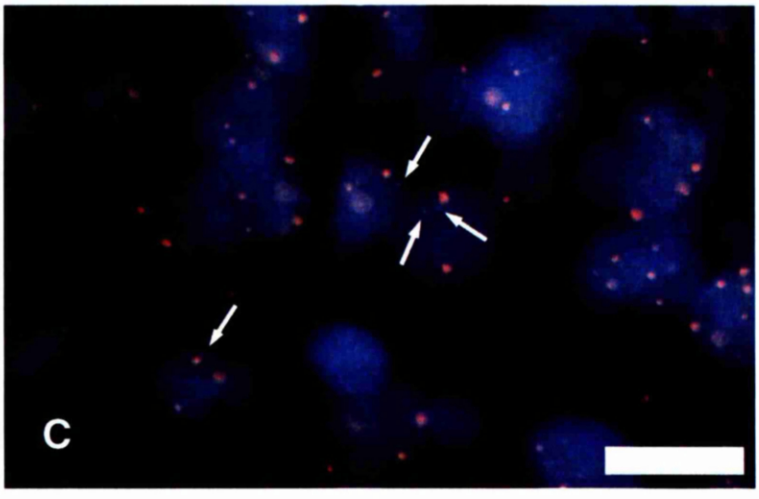
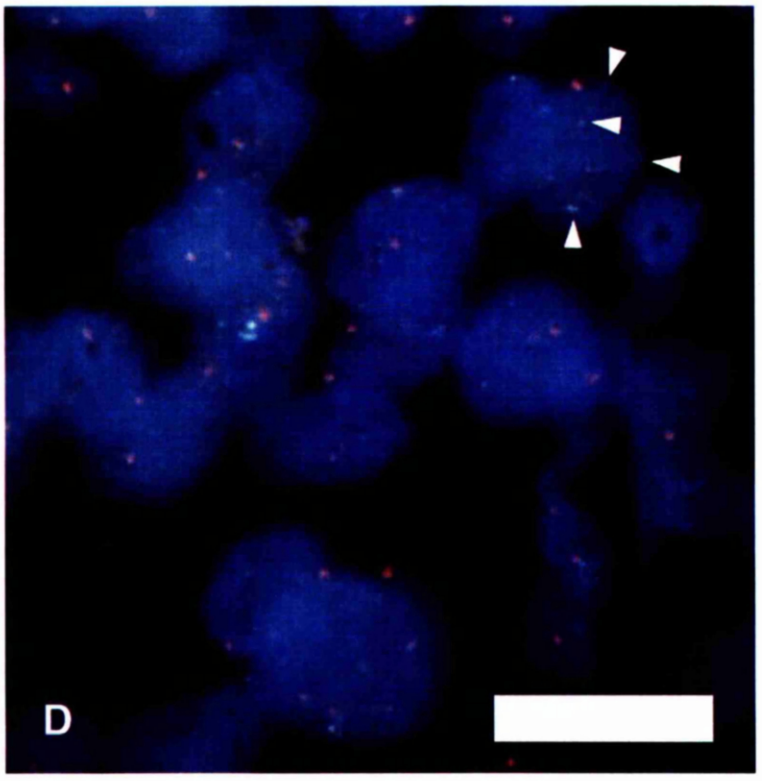
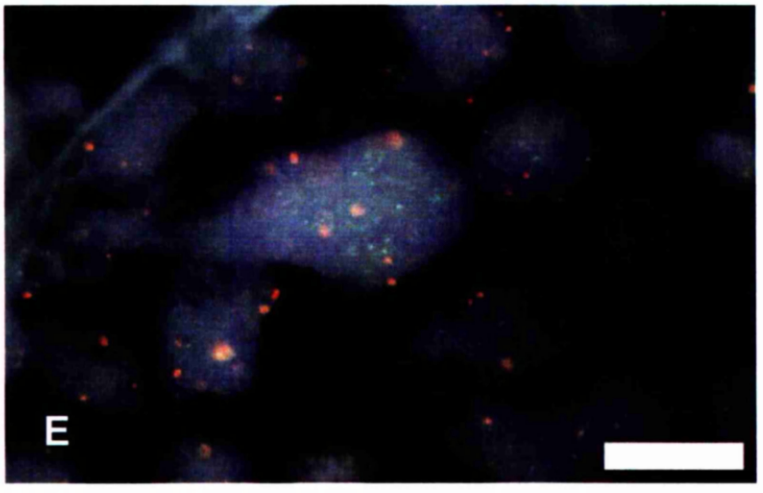

C


D


E

3.5.2 Assessment of Tissue for Adequate Digestion

As outlined in Section 3.3.2, proteolysis is required for *in situ* hybridisation on formalin fixed tissue, to access the DNA. However this must be achieved with minimal tissue damage, to enable morphology to be correlated with genetic aberrations. Optimum maintenance of cell and tissue morphology following unmasking of nucleic acids is achieved by assessment of tissue sections following proteolytic digestion prior to hybridisation. Viewing the nuclear staining intensity due to the intercalation of DAPI with DNA is a reliable indicator of the extent of digestion. Areas from distinct regions of the tissue sections were assessed to control for variability in fixation effects and thus proteinase K digestion. If the digestion of at least two thirds of the tumour was optimal, then these slides were suitable for hybridising. Figure 22A shows an underdigested TCC, figure 22B an optimally digested TCC and Figure 22C an over digested section of TCC. As would be expected with the wide range of tissue included, from small biopsies to cystectomies there was variability in the digestion times because of fixation differences.

3.5.3 Assessment of Control Sections

In every control section, 200 nuclei were assessed, both in the initial evaluation and when controls were included in the assessment of the patients' TCC's. The data from one control section are shown in Table 8. The mean copy numbers for chromosome 8 and *NAT2* were calculated as outlined in Section 2.4.8 and further explained in the accompanying legend. From this could be derived the data for each section and a mean value for normosomy established (Table 9).

Figure 22: Digestion Profile

The photomicrographs illustrating the variability in digestion are shown on the following page, magnification X 128, size bars 50μm.

22A: Underdigested TCC Section

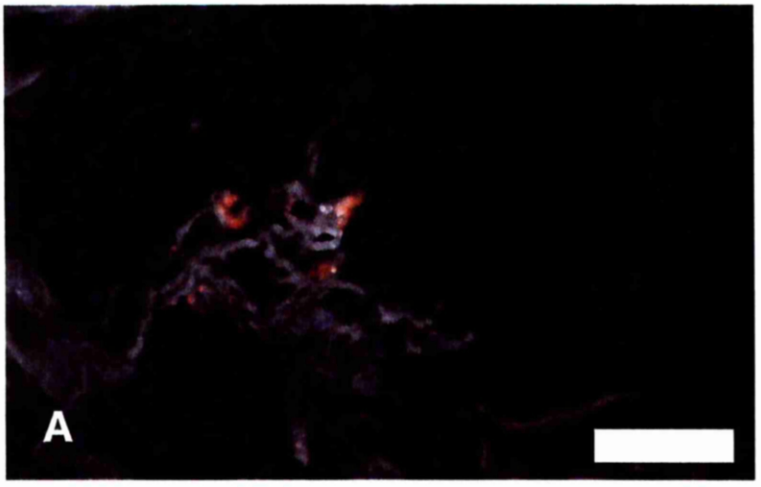
Although the nuclei can be discerned, protein remains which masks the preparation

22B: Optimally Digested TCC

Nuclei are clearly seen in this preparation, and this represents an optimally digested tissue section.

22C: Overdigested TCC

The morphology of the specimen is beginning to disappear, and nuclear borders are irregular, representing an overdigested tissue section.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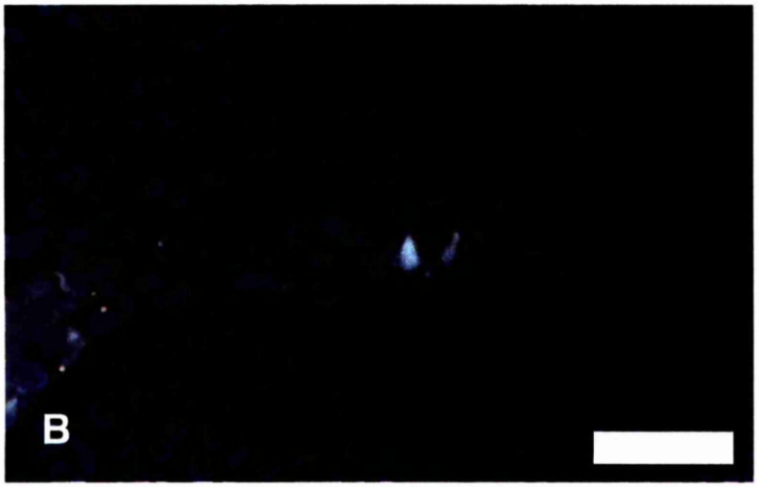

B

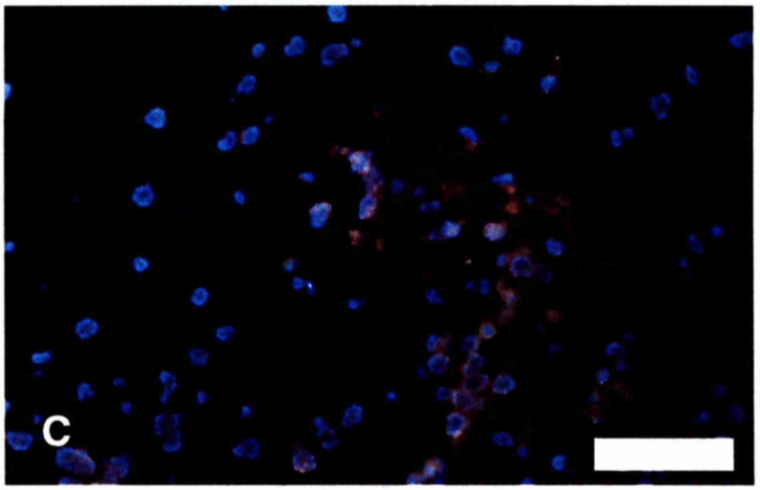

C

Table 8 Data For One Control Section

Observer	Probe	Signal (%)				Mean		
		0	1	2	MCCN/ MGCN	MCCN/MGCN	IOV (%)	Mean *NAT2*:8 (%)
A	8	11	30	59	1.48			
B	8	8	43	49	1.43	1.45		
A	*NAT2*	12	43	45	1.33			
B	*NAT2*	26	41	33	1.08	1.20	14.7	82.9

Results of one control section assessed by two observers (A and B), independently

Abbreviations: MCCN = mean chromosome copy number calculated by multiplying the number of nuclei with 0 signals by 0, the number of nuclei with one signal by 1 *etc* summing the number of signals and dividing by the number of nuclei counted. Therefore for observer A and the chromosome 8 probe, (11 X 0) + (30 X 1) + (59 X 2) = 148. Divide the number of signals by the number of nuclei (100) to give a value of 1.48.

MCGN = mean gene copy number, calculated in the same way as MCCN; The mean value was calculated by adding the two values together and dividing by 2.

IOV = interobserver variation, the difference between observer for the values obtained per signal; mean *NAT2*:8 is calculated by dividing mean MGCN by mean MCCN and expressing this as a percentage.

Assessment of 10 control sections with two independent observers established normal values for chromosome and cosmid probes (Table 9). For chromosome 8, the mean value of the data for MCCN (mean chromosome copy number) were 1.49 $\pm$0.07 (1.40-1.60), for MGCN (mean gene copy number) for *NAT2* 1.10 $\pm$0.20 (0.72-1.35); mean *NAT2*:8 ratio was 73.3 $\pm$ 13.1 (51.1-89.7); $\pm$SD, range in parentheses. A loss of 8 was defined as the MCCN minus 3 standard deviations *i.e.* 1.28, and a gain of 8 defined as the mean plus 3 standard deviations *i.e.* 1.70. The same calculation was applied to the MGCN for *NAT2* copy number and a loss was calculated as less than 0.50, a gain as

greater than 1.70. Once again the mean $\pm$ 3 SD was applied to the *NAT2*:8 ratio. Therefore a normal ratio was between 33.9% and 112.7%. These values were compared with the results from the tumours. Eighty percent of the controls and thirteen (35.1%) of the 37 TCC's were dual scored.

Table 9 Data for Ten Control Sections

Chromosome 8 MCCN	Cosmid *NAT2* MGCN	*NAT2*:8 ratio (%)
1.45	1.20	82.8
1.53	1.32	86.3
1.60	0.97	60.5
1.40	0.72	51.1
1.51	1.35	89.6
1.42	1.06	74.4
1.55	0.92	59.3
1.51	1.27	84.4
1.51	1.03	67.6
1.40	1.08	77.1

The results of the evaluation of 5 control sections and two observers is shown in Table 10. This exemplifies the methodology used, initially for control material and then applied to carcinomas in the patient series. Calculations were as previously outlined in Table 8 and in Section 2.4.8.

Abbreviations: MCCN/MGCN = mean chromosomal copy number/ mean gene copy number; mean *NAT2*:8 = mean ratio of *NAT2*:8 (%)

Table 10 Results of Evaluation of 5 Control Sections and Two Observers

Slide	**1**		**2**		**3**		**4**		**5**	
Observer	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
MCCN 8	1.48	1.43	1.63	1.57	1.38	1.43	1.61	1.41	1.47	1.59
MGCN *NAT2*	0.94	1.13	0.95	0.98	0.77	0.67	1.33	1.38	0.94	1.13
Mean 8	1.53		1.60		1.40		1.51		1.53	
Mean *NAT2*	1.03		0.97		0.72		1.35		1.03	
IOV 8	5.7		2.7		2.4		9.3		5.7	
IOV *NAT2*	13.0		2.5		10.2		2.6		13.0	
Mean *NAT2*:8	67.6		60.5		51.1		89.6		67.6	

This table details the scoring information derived from the assessment of 5 bladder sections and two observers.

Abbreviations: MCCN, MCGN as previously defined inTable 8, IOV=interobserver variation (%); the ratio of *NAT2*:8 expressed as a percentage.

3.5.4 Inter Observer Variation

Inter observer variation (IOV), a measure of reproducibility of the technique, for controls was 6.97% ± 6.05% for chromosome 8 and 11.79% ± 6.04% for *NAT2*. IOV for the tumours for chromosome 8 was 5.75%± 4.41, for *NAT2* 10.95%± 18.18. Figures represent the mean ±SD (Table 11). High inter observer variation was noted when the *NAT2* copy number was very low, but the scores were still concordant. The data from which these figures were derived is shown in the table below.

Table 11 Figures for IOV in Controls and Tests

Section	IOV chromosome 8	IOV *NAT2*
Control	2.4	10.2
Control	2.5	9.3
Control	15.9	20.5
Control	16.8	5.9
Control	5.7	13.0
Control	7.2	18.2
Test	9.6	63.1
Test	3.0	41.9
Test	3.5	27.9
Test	1.6	14.1
Test	3.2	6.3
Test	3.6	10.9
Test	5.1	4.5
Test	13.8	3.6
Test	13.6	20.8
Test	5.8	2.2
Test	1.0	23.9

This table shows the data obtained from the evaluation of tissue sections both in control and test sections from two observers. IOV (inter observer variation) is a measure of concordance between observers.

3.5.5 Evaluation of TCCs

Each TCC case was stained with haematoxylin & eosin, compared with the FISH stained section and specific tumour areas marked for analysis (Figure 13). The section was also assessed for variations in pathology. For example, where a TCC had been reported as superficial in part of the tissue but muscle-invasive in another, both areas were scored. If more than one tissue fragment was present in a biopsy specimen, each

Table 13 Stage and Grade of Recurrent TCCs with FISH Results

Patient	MCCN Chromosome8	MGCN Cosmid *NAT2*	*NAT2*:8 ratio (%)	Stage and grade
RNP1(2)	1.67	1.18	70.8	pT1G3
RNP1(3)	1.51	0.88	58.3	Severe dysplasia
RNP2(2)	1.59	1.30	82.4	PTaG1
RNP2(3)	1.57	1.09	69.6	PTaG1
RNP3(2)	1.64	0.73	44.6	PTaG2
RNP3(3)	1.62	0.94	58.0	PTaG2
RNP4(2)	1.66	1.01	60.6	PTaG2
RNP4(3)	1.75	1.27	73.0	pT1G2
RP1(2)	2.13	1.38	65.0	pT1G3
RP1(3)	2.36	1.06	46.2	PTaG2
RP1(4)	3.17	2.69	84.8	pT2G3
RP2(2)	1.54	0.53	34.4	PTaG3
RP2(3)	1.64	0.68	41.5	pT2-4G3
RP3(2)	2.38	0.49	20.4	PTaG2
RP3(3)	2.49	2.03	81.5	pT1G3
RP3(4)	2.17	1.37	63.1	pT2G3
RP4(2)	3.61	1.72	47.6	pT2G3,CIS
RP4(3)	3.27	2.30	70.4	pT2G3,CIS

This table shows the results derived from assessment of FISH stained TCCs, together with stage and grade.

Abbreviations: MCCN= mean chromosomal copy number; MGCN= mean gene copy number; *NAT2*:8= the ratio of *NAT2*:8 as a measure of hybridisation efficiency expressed as a percentage; RNP=recurrer non-progressor, number in parentheses refers to recurrence; RP=recurrer progressor, number in parentheses refers to recurrence (2, 3), progression (4); except for RP2 and 4 where recurrence 3 was also the progression event.

3.5.5.3 Assessment of Mean Chromosome and Gene Copy Number

Seventeen tumours were normosomic by the definition of MCCN/MCGN outlined in section 3.5.3 (Figure 21B, *NAT2* signals arrowed). Twenty tumours were abnormal for gene and/or chromosomal copy number (Tables 12 and 13). One (3%) had loss of *NAT2* and normosomy 8; two (6%) had loss of *NAT2* with polysomy 8; ten (27%) had normosomy *NAT2* and polysomy 8 (Figure 21C, *NAT2* hybridisation signals arrowed) and seven (19%) had polysomy of both gene and chromosome (Figures 21D, *NAT2* arrowed, and 21E). Comparing grade with any abnormality, using the chi square test, two of eight grade 1, 5 of 11 grade 2 and 14 of 17 grade 3 were abnormal (p=0.01). Comparing stage with any abnormality, 7 of 18 were pTa, 5 of 8 were pT1 and 9 of 10 were pT2 and above (p=0.03).

3.5.5.4 *NAT2*:8 Ratio

The *NAT2*:8 ratio expressed as a percentage was also used in the assessment of the TCC's.But there were only two tumours categorised as having loss of *NAT2* over and above those defined by MGCN. But in some cases, *NAT2*:8 ratio may be a more accurate measure than mean gene copy number of gene dosage as it takes chromosome copy number into account in the analysis. That is, a polysomy 8 could have a "normal" gene copy number such as patient NR6 (Table 12) which is defined as a loss by gene to chromosome ratio. Both methods of analysis were included in this study; either gave valuable information which may have been missed by including one or the other in the assessment of gene copy number.

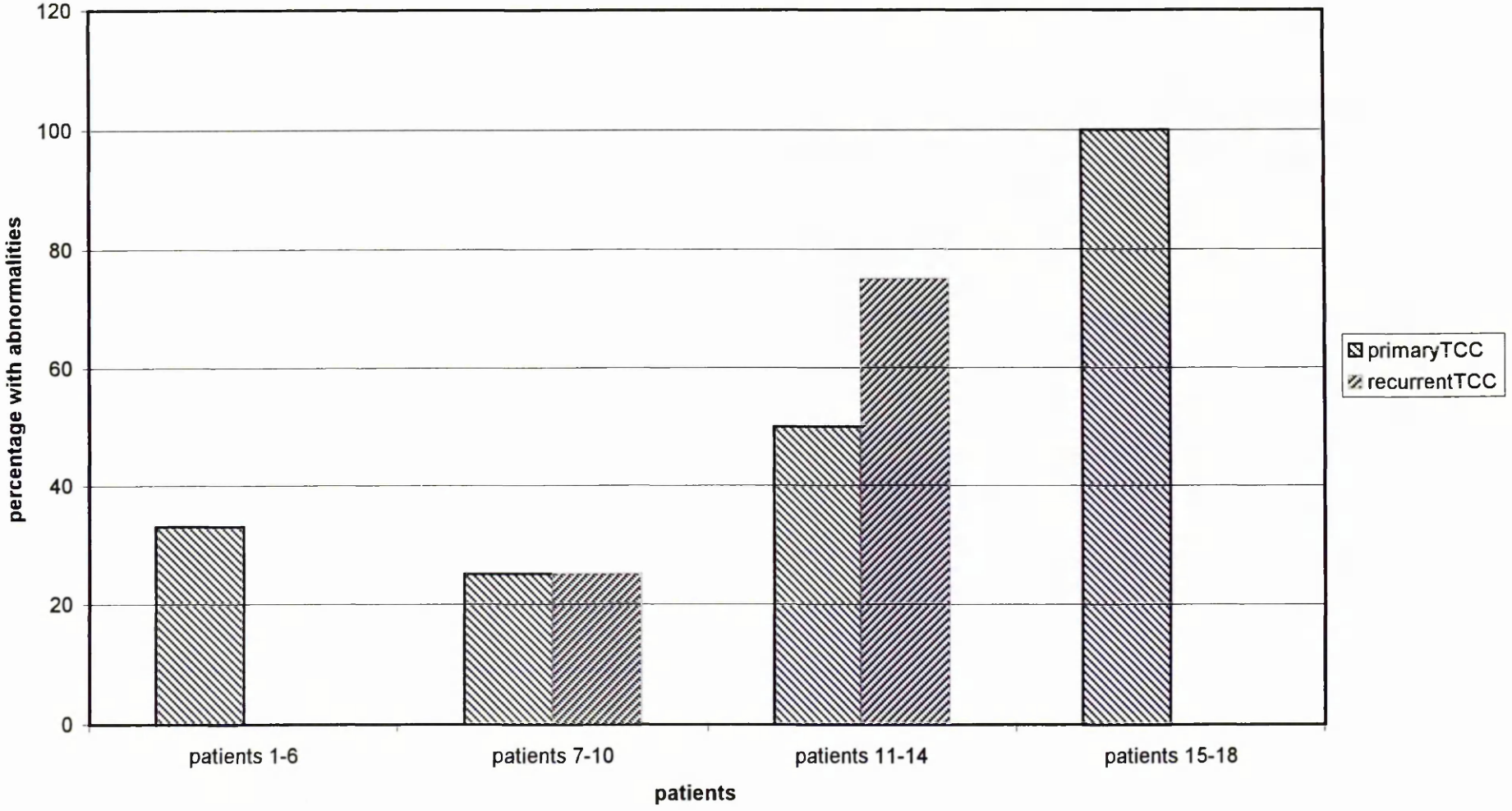
A normal ratio was defined as within the range 33.9% to 112.7%, following assessment of 10 tissue sections normosomic for chromosome 8 and *NAT2* copy number (Table 9). Thirty two tumours had a normal ratio, 5 were abnormal, defined as loss of *NAT2* copy number relative to chromosome 8 copy number. Two defined as normosomic for *NAT2* (NR6 and PP3) had a loss of *NAT2* by this method, the remaining 3 had a loss of *NAT2* defined either by mean gene copy number or mean ratio.

The tumour reported as severe dysplasia was normal for both gene and chromosome copy number using either MCCN/MGCN or ratio of *NAT2*:8 to define normosomy.

Figure 23: Percentage of Abnormal TCCs in each Patient Group

This graph illustrates the percentage of abnormal tumours categorised by patient group and is shown on the following page.

Figure 2: Percentage of Abnormal TCCs for each Patient



3.5.6 Assessment of Primary TCCs

Nineteen patients were included in the study (Table 12).

3.5.6.1 Mean Copy Number For Chromosome 8

Nine patients were normosomic for chromosome 8 as previously defined. Ten patients had polysomy of chromosome 8. Comparing stage with copy number, 7 of 10 pTa were normosomic, 3 were polysomic; 2 of four pT1's were normosomic, two were polysomic; no pT2 or above were normosmic, 5 of 5 were polysomic for chromosome 8. Comparing grade with copy number, 4 of 6 grade 1s were normosomic, 2 polysomic; 4 of 5 grade 2s were normosomic, one was polysomic and 1 of 8 grade 3 patients were normosomic, 7 were polysomic (Table 14).

Table 14 The Number of Index TCCs Normosomic & Aneusomic for Chromosome 8

Stage/grade	Chromosome 8	
	Normosomic	Polysomic
pTa/pT1	9	5
pT2 and above	0	5
Grade 1	4	2
Grade2/3	5	8

The number of index (primary) TCCs normosomic and polysomic for chromosome 8 copy number categorised by stage and grade.

3.5.6.2 Copy Number for *NAT2* in Primary TCCs

Fourteen patients were normosmic for *NAT2* copy number, five aneusomic, as previously defined. Two patients TCCs demonstrated a loss of *NAT2*, three showed an increase in *NAT2* copy number. Comparing stage with copy number, 8 of 10 pTa TCCs were normosomic, two aneusomic, both showing a loss of *NAT2* copy number; 3 of 4 pT1s were normosomic, one aneusomic, demonstrating an increase and 3 of 5 pT2 or above were normosomic, two aneusomic, both with an increase of gene copy number. Comparing grade with copy number, 5 of 6 were normosomic for grade 1, one had a loss of *NAT2* copy number; 4 of 5 were normosmic for grade 2 TCCs one demonstrated a loss; 5 of 8 grade 3s were normosmic, 3 had a gain of copy number (Table 15).

Table 15 The Number of Index TCCs Normosomic and Aneusomic For *NAT2* Copy Number

Stage/grade	*NAT2*	
	Normosomic	Aneusomic
pTa/pT1	11	3
pT2 and above	3	2
Grade 1	5	1
Grade2/3	9	4

The number of index TCCs normosomic and aneusomic for *NAT2* copy number categorised by stage and grade.

3.5.6.3 Copy Number for Chromosome 8 and *NAT2*

Using the MCCN/MGCN to assess primary TCCs, eight were normosomic and 11 aneusomic for chromosome and/or gene copy number (Table 16). Abnormalities of either chromosome or gene copy number were seen in 2/6 (33%) of grade 1 TCCs and 9/13 (61.5%) of grade 2 or 3 TCC. There was an association with high stage and aneusomy, however, as 6/14 (36%) of pTa/pT1 TCCs were abnormal whereas 5/5 (100%) of pT2 or above were abnormal. The percentage of each patient group with genetically abnormal primary and recurrent TCCs is illustrated in Figure 23. Normosomy was observed in 4 of 6 NR patients, 3 of 4 RNP patients, 1 of 4 RP patients but in none of the PP patients.

Table 16 The Number of Index TCCs Normosomic and Polysomic For Chromosome 8 and *NAT2* Copy Number

Stage/grade	Chromosome 8 & *NAT2*	
	Normosomic	Aneusomic
pTa/pT1	8	6
pT2 and above	0	5
Grade 1	4	2
Grade2/3	4	9

The number of index TCCs normosomic and aneusomic for chromosome 8 and *NAT2* copy number categorised by stage and grade

3.5.6.4 *NAT2*:8 Ratio

Applying the ratio of *NAT2*:8 to categorise primary TCC's, fifteen had a normal ratio, as previously defined, 4 were abnormal, of whom 1/6 were grade 1, 3/13 grade 2 or 3. Four of 14 were pTa or pT1 and 1 of 5 were pT2 or above. (Table 17).

Table 17 The Number of Index TCCs Normosomic and Aneusomic For *NAT2*:8 Ratio

Stage/grade	*NAT2*:8 Ratio	
	Normosomic	Aneusomic
pTa/pT1	11	3
pT2 and above	4	1
Grade 1	5	1
Grade2/3	10	3

The number of index TCCs normosomic and aneusomic for *NAT2*:8 ratio categorised by stage and grade

3.5.7 Recurrent Tumours

Eighteen recurrent TCCs from the patient groups, RNPs and RPs, were studied for abnormal *NAT2* or chromosome 8 copy number (Table 13).

3.5.7.1 Copy Number of Chromosome 8

Nine of eighteen recurrent tumours were polysomic, as previously defined. Comparing stage to copy number, 6 pTas were normosomic, whilst 2 were polysomic; one of 4 pT1s was normosomic, 3 polysomic; one of 5 pT2 and above was normosomic, 4 were polysomic. Comparing grade with copy number, 2 of 2 grade 1 tumours were normosomic; 3 of 6 grade 2s were normosomic, 3 were polysomic and 3 of 9 grade 3 TCC's were normosomic, 6 were polysomic (Table 18).

Table 18 The Number of Recurrent TCCs Normosomic and Polysomic For Chromosome 8 Copy Number

Stage/grade	Chromosome 8	
	Normosomic	Polysomic
pTa/pT1	7	5
pT2 and above	1	4
Grade 1	2	0
Grade2/3	6	9

The number of recurrent TCCs normosomic and polysomic for chromosome 8 copy number categorised by stage and grade

3.5.7.2 *NAT2* Copy Number

Thirteen recurrent TCCs were normosomic for *NAT2*, five were aneusomic. Comparing stage to copy number, 7 of 8 pTas were normosmic, one showed a loss; 3 of 4 pT1s were normosomic, one showed an elevated copy number and 2 of five pT2 and above were normosomic, 3 were aneusomic, all showing an elevated copy number. Comparing grade to copy number, 2 grade 1s were normosomic, none was aneusomic; 5 grade 2 were normosomic, one aneusomic, showing a loss of *NAT2* copy number and 5 of 9 grade 3s were normosmic, 4 aneusomic, all with an elevated copy number (Table 19).

Table 19 The Number of Recurrent TCCs Normosomic and Aneusomic For *NAT2* Copy Number

Stage/grade	*NAT2*	
	Normosomic	Aneusomic
pTa/pT1	10	2
pT2 and above	2	3
Grade 1	2	0
Grade2/3	10	5

The number of recurrent TCCs normosomic and aneusomic for *NAT2* copy number categorised by stage and grade

3.5.7.3 Chromosome 8 and *NAT2* Copy Number in Recurrent TCCs

Using the MCCN/MGCN to assess copy number, the last documented recurrence in RNP 4 showed increase of copy number for chromosome 8 although this was at the low end of polysomy (1.75); all other patients' recurrences in the RNP group were normal for gene and chromosome copy number. Three of the four recurrer progressor patients (RP) were abnormal for either gene or chromosome copy number (Tables 13 and 20). For example, patient RP1 who had polysomy of chromosome 8 and loss of *NAT2* in the primary TCC remained polysomic for 8, but first and premuscle-invasive events were normosomic for *NAT2*. The muscle-invasive tumour showed polysomy for 8 and elevated copy number for *NAT2*.

Table 20 The Number of Recurrent TCCs Normosomic and Aneusomic For Chromosome 8 & *NAT2* Copy Number

Stage/grade	Chromosome 8 & *NAT2*	
	Normosomic	Aneusomic
pTa/pT1	7	5
pT2 and above	1	4
Grade 1	2	0
Grade2/3	7	8

The number of recurrent TCCs normosomic and aneusomic for chromosome 8 and *NAT2* copy number categorised by stage and grade.

In summary, eight out of nine (89%) of patients with muscle-invasive transitional cell carcinoma (TCC) at presentation or who developed this pathology (RP and PP groups) had aberrant gene and/or chromosome copy number at some point in their progression from superficial TCC to locally invasive TCC to muscle-invasive TCC. Whereas 4/10 (40%) of patients with non-invasive disease or locally invasive TCC (NR and RNP groups) had aberrations.

3.5.7.4. Ratio of *NAT2*:8

One recurrent tumour from patient RP3, already defined as having loss of *NAT2*, had loss with *NAT2*:8 ratio, therefore this calculation gave no additional information over and above mean gene copy number.

3.5.8 Tumour Heterogeneity

Five TCCs exhibited heterogeneity, and in those cases, the most aneusomic cell population was used in the final analysis. One of these was from the index TCC (pTaG1) of a RNP patient. This appeared to be a clone of aberrant cells amidst a normosomic population. Previously this patient's TCC had been assessed with probes for other chromosomes (Table 21) and again this particular area had been abnormal. The other four TCCs were from patients with muscle-invasion at presentation (n=1) or who developed this pathology (n=3). In addition, two separate tissue sections analysed from

one RP patient's cystectomy specimen had different genetic profiles. One was normosomic, the other aneusomic, and again the most aneusomic cell population was used in the final analysis.

3.5.9 Association with Other Chromosomal Abnormalities

Some of the index TCCs in this study had previously been investigated for chromosomal abnormalities common in bladder cancer. The results were compared with those of chromosome 8 and *NAT2*. For simplicity, the combination of mean gene and chromosome copy number only was compared, as shown in Table 21. In the NR group, one patient with a loss of *NAT2* as defined by mean gene copy number was normosomic for the other chromosomes. The NR with polysomy 8 in this study had been polysomic for all other chromosomes studied. This strongly suggests that polyploidisation has occurred and that this was possibly the mechanism for carcinogenesis to occur. One of 4 RNP patients had abnormalities in other chromosomes, RNP3 had polysomy of 8 in one tumour area only. This area had shown aneusomy in all of the other chromosomes analysed, whilst the remaining tumour areas demonstrated normosomy. This indicates development of a malignant clone within an essentially normosomic tumour for the chromosomes analysed. However monosomy of chromosome 9 was also present again within this one area which may be the initiating event in this patient's TCC. All of the RP's had abnormalities of at least one other chromosome, one of whom had abnormalities in 3 other chromosomes (RP4).

3.5.10 Summary

Figures for normal gene and chromosome copy number (MCCN/MGCN) and mean *NAT2*:8 ratio were established with control sections. Thirty seven tumours were then assessed, from 19 patients. Using MCCN/MGCN, twenty were abnormal, whereas 5 with mean *NAT2*:8 ratio were abnormal. In either case a significant proportion of the TCCs studied were abnormal, and patients with muscle-invasive carcinoma at some point in their clinical history had a higher proportion of abnormalities that those who did not have this pathology.

Table 21 Association with Other Chromosome Abnormalities

Patient	Chromosome					
	7	8/*NAT2*	9	10	11	17
NR1	N	N	N	N	N	N
NR2	N	N	N	N	N	N
NR3	N	N	N	N	N	N
NR4	N	**N/L**	N	N/D	N	N
NR5	N	N	N	N	N	N
NR6	**G**	**G/N**	**G**	**G**	**G**	**G**
RNP1	N	**N**	N	N/D	N/D	N
RNP2	N	N	N	N	N	N
RNP3	**G**	**G/N**	**L**	N	**G**	**G**
RNP4	N	N	**L**	N/D	N/D	N
RP1	N	**G/L**	**L**	N	N/D	N
RP2	N	**N**	N	N	N	**G**
RP3	N	**G/N**	N	N/D	N/D	**G**
RP4	**G**	**G/G**	N	N/D	**G**	**G**

A comparison of genetic and chromosomal abnormalities observed in this study, in the index TCC's compared to the results of previous research with DNA probes for chromosomes commonly abnormal in bladder cancer.

Abbreviations: NR = non recurrer, RNP = recurrer non progressor, RP= recurrer progressor, G = gain of chromosome copy number as previously defined (Watters *et al*, 1999); N = normosomy, as defined in Section 3.5.3; L = loss (monosomy) as previously defined (Bartlett *et al*, 1998); N/D=not done.

CHAPTER 4 DISCUSSION

4.1 Introduction

The rising incidence in the developed world for cancer reflects statistics for bladder carcinoma in Scotland (Figure 1), with no evidence that this trend will decrease well into the next century. The repeated observations that two primary TCCs with identical clinical and pathological profiles may have widely differing prognoses have led to the hypothesis that there are genetically distinct routes to recurrence and/or progression (reviewed by Foresman & Messing, 1997). Several genetic aberrations have been identified in TCC of the bladder and models for progression and to a lesser extent recurrence have been proposed (Dalbagni *et al*, 1993, Reznikoff *et al*, 1996). However few studies have emphasised the need for clinical follow-up of patients whose genetic aberrations have been studied, (for example those of Pycha *et al*, 1997 and Bartlett *et al*, 1998) therefore there is less known of the genetics of recurrence.

Several factors *e.g.* the depth of invasion, multifocality, high grade and size at presentation are associated with muscle invasion (Adshead *et al*, 1998). Currently pathologically determined factors (stage and grade) are the primary prognostic variables that dictate treatment strategies (Stein *et al*, 1998). However, there are still insufficient clinical and histological parameters to predict with accuracy which pTa or pT1 carcinomas will progress to detrusor muscle invasion. Increasingly the potential for combining molecular profiling with pathological and clinical diagnoses is being realised (Adshead *et al*, 1998, Knowles, 1998). Facilitation of selection of patients who may benefit from less or more aggressive treatments depending on the combination of their clinical, pathological and biological profiles is the ultimate goal of an increasing number of diagnosticians.

4.1.1 Patient Management

Patients who present with non-muscle invasive (pTa/pT1) carcinoma routinely have the tumour removed surgically, but 50-70% of such patients will present with recurrent carcinoma within 1-2 years (van der Meijden, 1998). Many patients are in fact undertreated at presentation of their primary carcinoma. In an attempt to prevent this some centres treat adjuvantly with intravesical drugs, and reduced rates of recurrence have been reported (Oosterlink *et* 1993, Tolley *et al*, 1996). However, there remains a clinical dilemma as the patients who do not recur have had unnecessary chemotherapy;

in this context a means of identifying patients who are at highest risk of recurring would be beneficial.

The accumulation of data supporting the theory that carcinogenesis is accompanied by several genetic anomalies (Nowell, 1976) has led to proposals of genetic mechanisms of recurrence (Pycha *et al*, 1997, Harnden *et al*, 1999, Watters *et al*, 1999). These changes give insight into potential ways to improve the targetting of patients for more aggressive treatment regimes. This aspect will be discussed in more detail with particular reference to the results of this study later in the discussion.

In this study only patients presenting with papillary TCC were included, as the molecular biology of CIS differs from papillary TCC (Reznikoff *et al*, 1995). Evidence is emerging that pTa and pT1 are also genetically distinct (Richter *et al*, 1997, Sauter & Mithatsch, 1998). Foresman & Messing, (1997) in their review quote results of a study of 249 pTa or pT1 carcinomas where patients were followed up for a median period of 39 months. Four percent of pTa compared with 30% of pT1 patients progressed and of grade 1 pTa/pT1 lesions only 2% progressed to muscle invasive/metastatic disease compared with 11% of grade 2 and 45% of grade 3 carcinomas. Progression occurred within 25 months in most cases. Nearly 50% of pT1G3 carcinomas progressed over this interval. These results support the generally accepted view by urologists who treat pTaG1 TCC as a completely distinct disease entity from pT1G3 TCC. pTaG3 and pT1G1 carcinomas are unusual and pT1 carcinomas tend to be more aggressive (of higher grade) than pTa lesions. As it is far more common to have a pT1G3 lesion than a pT1G1 lesion, high grade may be the significant factor as this is known to be associated with aggressive tumours.

Rather than grade or stage being of greater import, the combination of the two may be the driving factor in tumour aggressiveness. Once a malignant cell has invaded the basement membrane the characteristics of a grade 3 tumour, by definition showing more features of malignancy than a grade 1 may drive the cell to become invasive. However the argument remains flawed as not every pT1G3 becomes detrusor muscle invasive and some pTaG1 carcinomas do develop invasive profiles. This is borne out in the patient cohort used in this study as a diagnosis of a pT1G3 carcinoma at presentation, in the patient groups analysed here, did not affect disease outcome, as one "non recurrer", one "recurrer non progressor" and one "recurrer progressor" had this diagnosis. Of those that died four were grade 1, two were grade 2 and one grade 3 at presentation. From assumptions made on disease course based on diagnosis at

presentation, the higher grades should be associated with more deaths. However these patient groups were very small, precluding any statistical associations with stage, grade and clinical behaviour. In this small patient cohort, the stage and grade of the primary carcinoma was not associated with individual outcome, even if this was a pT1 grade 3, despite reports such as that of Foresman and Messing (1997).

Within this study, 5 patients with pT2 or above (muscle invasion) at presentation were included. From previous research (Knowles, 1998) non-muscle invasive and muscle invasion at presentation have differing clinical and pathological outcomes. The latter is also associated with more genetic aberrations and other biological abnormalities (Knowles, 1998). A comparison of the genetic anomalies of chromosome 8 and *NAT2*, in pTa versus pT1 versus pT2 and above, combined with non recurrent through to progressed at presentation disease has the potential to identify chromosome 8/*NAT2* status as a biological marker of recurrence or progression.

4.1.2 Exposure to Chemical Carcinogens

4.1.2.1 Introduction

This study investigated the genetics of the aromatic amine metabolising enzyme, NAT2, thus industrial history or smoking was considered, both of which involve exposure to aromatic amines.

4.1.2.2 Industrial Exposure

The lack of information available on patients' occupational history in this study reflects the low priority accorded to such documentation in routine clinical practice. Ten of the 19 patients had occupational histories documented at initial admission, but this was not necessarily recorded at the Urology clinic. This is surprising, as bladder cancer is one of the few neoplasms directly correlated with industrial exposure (Case *et al*, 1954, Risch *et al*, 1995). A study to assess the accuracy of recording occupational history was carried out by McCahy *et al*, (1997). Despite using a specially designed questionnaire, accuracy was achieved in only 39% of cases. The researchers argue that this lack of information could lead to missed claims for compensation and under-reporting of occupational mortality.

A five year field survey by Case *et al*, (1954) was the first study to connect the causal link of potential chemical carcinogens with bladder cancer, prompting studies relating bladder cancer aetiology with exposure to chemical carcinogens to be carried out. For example, Cartwright *et al*, (1982) studied a group of employees in dye

manufacturing and found a high proportion with bladder cancer and the slow acetylation phenotype. With the decline in manufacture of dyes and other aromatic amines in Britain and other Western countries since the 1970's, (Risch *et al*, 1995, Golka *et al*, 1996), awareness of environmental exposure has lessened. However risk factors remain for those involved in textile, rubber and leather industries, and workers in dyestuff production still experience a relative bladder cancer risk of 2.4 to 2.6, even after adjustment for smoking and age (Sørlie *et al*, 1998).

Screening exists for workers in high-risk occupations, specifically urine screening programmes (Droller, 1985), that have a higher pick-up rate than the general population, despite the low sensitivity of this test. Regular cystoscopic monitoring in this high risk group has been suggested as they are approximately seven times more likely to develop TCC than the general population (Schulte *et al*, 1986). However, although this procedure may detect early cases of TCC, the latency of the development of TCC (Risch *et al*, 1995) means that individuals are subjected to many cystoscopies and anaesthetics before a positive result is detected. Individual anxiety would undoubtedly be heightened and the trauma involved would be uncomfortable. Also whether or not the resources exist to cope with the increased workload is questionable.

An alternative is to use a chemical reagent strip to test for haemoglobin, indicating haematuria (Goldstein & Messing, 1998). Almost all bladder cancers will cause haematuria at some point. In their review, Goldstein & Messing, (1998) reported results of two studies of the general population using haematuria home testing; in both studies, 15-20% of the screened populations had haematuria and 6 to 8% were found to have urothelial cancers. However, the disavantage of a home based test is that the responsibility is entirely on the patient, although the patient could be called to clinic, but this would considerably increase the clinician's workload. As is the argument for many screening programmes, such as the USA program for detecting prostate cancer, the problem remains resource based in a country such as the UK where private health schemes are rare and costly. Thus at present, occupational screening remains a urine based procedure even though urine cytology has a low sensitivity.

4.1.2.3 Environmental Exposure

With respect to TCC, the only direct causal link with carcinogenesis and environmental factors is through smoking. The high proportion of patients with a smoking history in this study reflects the statistics for Western populations. Cigarette smoke accounts for at least 50% of bladder cancers, and in particular the use of black

tobacco is more strongly associated with bladder cancer than lung cancer on a geographical basis (Vineis & Martone, 1996). In this study, twelve of 13 patients, for whom information was available, had a smoking history.

The two ways of curing tobacco change the colour of the resultant product. Black tobacco is air cured and blond tobacco is flue cured. Europeans tend to smoke black tobacco, in the USA the blond variety is more popular. Black tobacco has a higher concentration of carcinogens such as 4-aminobiphenyl and β naphthylamine compared to the blond variant. A significantly higher frequency of G:C to C:G transversions are found in bladder cancer compared to lung or colorectal carcinoma. These mutations may be induced by free oxygen radicals present in cigarette smoke (Knowles, 1998). Landman & Droller (1998) in their review suggest that the carcinogens caused by pyrolysis may act at a late stage in bladder cancer progression, perhaps by causing late chromosomal damage possibly by increased frequency of *p53* mutations. This may be more pronounced in patients who smoke black tobacco, with its higher concentration of toxic metabolites.

A study of the epidemiology of bladder cancer patients (Vineis and Martone, 1996) found that those who smoked black tobacco had a greater relative risk of developing bladder cancer compared to individuals who smoked blond tobacco (4.0 versus 1.8). The relative risks of developing bladder cancer were lessened in individuals who stopped smoking 2 to 15 years previously, (0.63 compared with 1.0 in smokers; Morrison *et al*, 1984). However, there was still a 2-3 fold increase in bladder cancer incidence if an individual had ever smoked versus non-smokers, (Morrison *et al*, 1984), regardless of the type of tobacco used. Higher grade and more frequent recurrences are also associated with TCC patients who are smokers (reviewed by Landman & Droller, 1998). Within this study no association with smoking and recurrence or grade was possible, however the high number of smokers in this cohort reflects previous research.

4.1.3 Follow-up

In four patients included in this study, length of follow-up available was reduced by factors such as the patient moving to another hospital or the original case notes being lost. Bladder cancer is characterised by recurrent disease over many years, requiring patients to attend clinic many times. This increases the risk of case notes going missing

or patients moving to another treatment centre. Thus these findings are fairly typical of patients who have long term follow-up.

4.1.4 Deaths Due to Bladder Cancer

Deaths due to bladder cancer all followed either recurrent progressive disease or muscle invasion at presentation. These results reflect those of Bartlett *et al*, (1998) and Watters *et al*, (1999). Patients were categorised in a similar way and only those in the recurrent progressive category died of bladder cancer, although patients with muscle invasive disease at presentation were excluded in those studies. Pryor *et al*, (1973) looked at factors that influence the survival of patients with TCC. Depth of invasion (stage) and tumour grade (degree of tumour differentiation), lack of lymph node invasion and patients age were most important. Three year survival rate in a cohort of 691 patients followed up over 10 years was 82% for T1 lesions, 48% for T2, 21% for T3 and 4% for T4 carcinomas. Higher grade carcinomas also had a shorter 3 year survival rate. The outcome in this patient cohort thus reflects the general trend for patients with aggressive carcinomas.

4.1.5 Conclusion

The detailed data gathered from the patient notes provided a basis for assessment of the results generated by the FISH assays, the aim of which was to correlate genetic aberrations with the clinical course of TCC.

4.2 Methodological Aspects

4.2.1 Introduction

Methodologies previously established in the laboratory for probe labelling and fluorescence *in situ* hybridisation were initially applied to the analysis of the TCCs in this study. However, there was limited experience in the use of a labelled unique sequence probe for FISH as applied to the study of genetic aberrations, especially in archival tissue sections. The preliminary experiments with nick translation to label the *NAT2* cosmid clone to produce a probe for FISH were unsuccessful. Modifications of both techniques were required to evaluate copy number in archival material. Several factors influence the intensity and specificity of the hybridisation signal; in particular the purity of the DNA probe and the size of the probe fragments used in the hybridisation reactions (Lichter *et al*, 1990). Both these factors are discussed below.

4.2.2 Nick Translation

4.2.2.1 Probe Production

A cosmid clone containing the *NAT2* sequence was synthesised following the method of Franke *et al*, (1994). The purity of the DNA probe was assumed to be acceptable, as the cosmid containing the *NAT2* sequence had been previously been labelled and applied successfully to the study of exfoliated cells in urine (Stacey *et al*, 1996) and to bladder barbotage cells (Stacey *et al*, 1999). The isolation of cosmid clones containing the sequence of interest has been a standard method for synthesising probes for fluorescence *in situ* hybridisation (Franke *et al*, 1994). Recently the production of probes by a different method has been reported (Davidson *et al*, 1998) to circumvent the difficulties of studying unique sequences in thin tissue sections (5 microns or less). Davidson *et al* (1998) constructed probes spanning the *MYC* and *EWS* regions, assembled from multimegabase contigs of yeast artificial chromosome clones (YAC) and removed *Alu* sequences by subtraction hybridisation. The probes were then labelled with biotin or fluorescein isothiocyanate and in subsequent hybridisation experiments yielded bright signals. Davidson *et al* (1998) also demonstrated the stability of the probes after multiple rounds of amplification by the polymerase chain reaction (PCR).

Probes have previously been produced by PCR as opposed to nick translation (Weier *et al*, 1994), by incorporating the label in the PCR, thus simultaneously

amplifying and labelling the DNA sequence. However, PCR is capable of amplifying a single molecule, thus specificity of the labelled DNA can be compromised (Stickland, 1992). In general the production of probes by nick translation is a simple procedure and the resulting fragments are of suitable size for *in situ* hybridisation. Methods such as that of Davidson and co-workers, (1998) claimed to produce an intense hybridisation signal but they were demonstrating chromosomal rearrangements flanking *MYC* and *EWS*, not aberrations of the sequence specific probe. However, it would be possible to buy the relevant YAC contigs and apply the methodology to the *NAT2* gene, and compare the results with the nick translation method. But for the purposes of this study, as will be discussed in the following section, with judicious modification of the existing nick translation protocol, probes were produced which were then successfully used in FISH.

4.2.2.2 Modifications of the Nick Translation Reaction

4.2.2.2.1 Introduction

Nick translation is an enzymatic labelling procedure that results in the uniform incorporation of label into DNA sequences which are of the appropriate size for *in situ* hybridisation (Leitch *et al*, 1994). The enzymes DNase I and DNA polymerase I incorporate labelled nucleotides into DNA. DNase I introduces single strand breaks (nicks) and DNA polymerase I catalyses the incorporation of new nucleotides. As the nicks are introduced at random into the double stranded DNA molecule, labelled fragments of different lengths and containing variable proportions of insert and vector are produced. There has been a suggestion made that the fragments anneal together via their overlapping portions to produce a probe network, thereby amplifying the target sequence *in situ* and enabling the nick translation method to achieve high sensitivity (Herrington & McGee, 1992). The end result is a DNA strand which has incorporated labelled nucleotide to produce a probe of between 200 and 600 base pairs, achieved by altering the relative concentrations of the two enzymes DNase I and DNA polymerase I (Leitch *et al*, 1994).

4.2.2.2.2 Optimising the Nick Translation Reaction

In order to ascertain the optimal nick translation reaction, several steps were taken to investigate ways in which the DNA fragment size could be altered within the reaction. Initially a simple comparison between two methods was made. The existing method produced unnicked cosmid whereas no product was visualised in the alternative protocol.

Comparison of enzyme concentrations revealed the alternative protocol contained a higher concentration of DNase I than the existing one. Titration of DNase I enzyme concentration produced fragments of acceptable length (Figure 16). Rigby *et al* (1977) noted that DNase I activity varies from different commercial sources. From the literature it appears that of the two enzymes DNA polymerase I and DNase I, DNase I is the more sensitive to factors such as prolonged storage, repeated freezing and thawing. It is also the enzyme required to initiate the nick translation reaction, therefore if the enzyme concentration is too low or the activity compromised, the reaction cannot proceed (Rigby *et al*, 1977). The extent of nucleotide replacement of the template can be controlled by varying the amount of DNase I in the reaction (Sambrook *et al*, 1988). From this evidence, it appears that DNase I concentration is a crucial parameter in the nick translation reaction. This was borne out by the results of the titration experiments, which finally incorporated an additional 4.8 mU/µl of DNase I into the reaction (Figure 16D).

4.2.2.2.3 Biotinylation

Biotinylated dUTP was added in the proportion 2:1 TTP:biotinylated dUTP. This allows for the most efficient incorporation of biotinylated dUTP into the DNA, as some steric hindrance is encountered due to the molecule's size (Figure 10). Incorporation of the biotin molecule at C11 or C16 allows the most efficient incorporation of biotinylated dUTP (Leitch *et al*, 1994). Initially in this study the protocol for radiolabelled probes was followed which recommended the addition of the 3 unlabelled nucleotides in the same proportion as the labelled nucleotide. However, radiolabels are much smaller than biotin and therefore incorporation of label is not compromised by size. This may have contributed to the failure of the initial experiment, but this parameter was not investigated to any extent as the results of the experiments varying DNase I concentration were successful. However because of the steric hindrance of biotin, unlabelled DNA was used in the initial comparative experiments (Section 3.3.2.2) and then reintroduced when the experiments were successful (Section 3.3.2.5). The experiment with biotinylated dUTP was then compared to the previous one without biotin; the addition of biotin did not appear to compromise the reaction. Therefore, no further modifications were undertaken.

4.2.2.3 Conclusion

The success of the nick translation reaction was essential to enable the probe for *NAT2* to be incorporated into FISH. A reliable, reproducible modification of a kit was developed which produced a DNA fragment of suitable size for FISH.

4.2.3 FISH

4.2.3.1 Preparation of the Tissue for FISH

Five micron tissue sections were cut from paraffin blocks. Contaminant DNA must be avoided, therefore gloves were worn throughout the section cutting and FISH technique. Nucleases present on the skin represented another possible source of contamination. Unfixed DNA or RNA can be degraded by nucleases, although this is less of a problem in formalin fixed material as fixed DNA or RNA are resistant to nuclease digestion (Warford, 1996). Intact tissue sections were used throughout the study, therefore areas of carcinoma could be assessed relative to non malignant regions within the same tissue section and regions of heterogeneity identified.

4.2.3.2 Modification of FISH Protocol for the Demonstration of Unique Sequence Probes in Paraffin Sections

4.2.3.2.1 Introduction

Fluorescent *in situ* hybridisation (FISH) is a technique involving the hybridisation of a known sequence of DNA or RNA into which is incorporated a suitable label (the probe) with target DNA or RNA. Aberrations of the gene or chromosome can then be evaluated following immunocytochemical detection with fluorescent labelled antibodies or directly if a fluorescent labelled probe is used. There are two disadvantages to using a directly labelled probe: the sensitivity is reduced when compared to indirectly labelled probes and there is no flexibility at the detection stage *e.g.* if a signal is weak the option to reapply the immunocytochemical steps is not possible, although this can introduce undesirable background staining. The advantage is that the technique is performed rapidly and the signal produced may be more intense. In this study, whereby loss of a unique sequence probe was anticipated, it was essential to use a method with the highest degree of sensitivity *i.e.* an indirect technique.

The established FISH protocol for commercial centromeric probes was applied to 5 micron tissue sections with the *NAT2* and chromosome 8 probes. This preliminary experiment showed that the technique was not readily applicable to the demonstration of

unique sequences in tissue sections as weak signal and precipitate (possibly non-specific binding of biotin) were observed. Therefore a number of modifications were introduced to increase signal intensity and decrease background staining. Throughout the changes introduced, emphasis on preservation of tissue morphology was maintained. One of the strengths of *in situ* hybridisation is that the conditions of pre and post hybridisation can be manipulated as necessary (Herrington & McGee, 1992).

4.2.3.2.2 Pretreatment and Proteolysis

Pretreatment and proteolysis of material enables the DNA to be accessed before *in situ* hybridisation can be performed and is essential for the success of the technique. Fixatives such as formalin are recommended for *in situ* hybridisation studies as these optimally preserve the tissue (Mitchell *et al*, 1992). Formalin is an aldehyde that forms cross links between protein molecules (Hopwood, 1996). For formalin fixed paraffin processed thin tissue sections, pretreatment and proteolysis involve dewaxing of the section, digestion with the appropriate proteolytic enzyme and denaturing of the target DNA. Pepsin or proteinase K are the most widely used enzymes for *in situ* hybridisation.

Several protocols for section pretreatment exist, before proteolysis, which are known as tissue permeabilisation steps. These include 0.2N HCl and Triton X (Hopman *et al*, 1992) or reducing agents such as sodium thiosulphate (Pauletti *et al*, 1996) and sodium thiocyanate, one of the strongest protein denaturing agents (Hopman *et al*, 1991). Some protocols combine 0.2N HCl and a reducing agent, such as the Pathvysion™ HER-2 DNA probe kit, (Vysis,UK). 0.2N HCl is thought to increase the hybridisation signals, as acid deproteinases the tissue thus increasing probe penetration (Syrjänen, 1992). This is due to the pH dependent fixation reaction that proceeds more rapidly at higher pH (Hopwood, 1996) and presumably is reversed following the introduction of an acid, such as HCl. Reducing agents break the protein disulphide bonds formed by formalin (Ottoway & Apps, 1984) and allow greater nuclear accessibility. Generally the use of a permeabilisation step reduces the proteolytic step thus causing less tissue damage. The inclusion of sodium metabisulphite is also thought to reduce autofluorescence when combined with proteinase K (Lowery, 1998).

Pepsin is primarily used as the proteolytic step in the demonstration of genomic DNA and some mRNA (Herrington & McGee, 1992) although proteinase K has also been used successfully (Pauletti *et al*, 1996). Pepsin in HCl had been previously used as the only pretreatment step in the demonstration of alpha and classical satellite probes in

formalin fixed paraffin processed thin sections (Watters, *et al*, 2000). This was not successful with the *NAT2* probe which led to an investigation of an alternative method. The nuclei appeared larger with the sodium bisulphite/proteinase K protocol, probably due to the permeabilisation step compared to the pepsin digestion alone. The variation of methodology in the literature (Murphy *et al*, 1995, Pauletti *et al*, 1996, Wagner *et al*, 1997) would suggest that some adaptation of an existing protocol is necessary when a new probe is used. Adapting the protocol in this study resulted in increased hybridisation intensity and lower background staining. This improved result, without compromising tissue morphology, was then applied to the study of chromosomal aberrations in TCC.

The essential step at this part of the FISH technique is to allow permeabilisation of the tissue for probe accessibility, with preservation of tissue architecture. Undoubtedly part of the technical variability, with regard to routinely formalin fixed tissues, is that there is no control over fixation length. This in turn will affect the pretreatment time, which may also result in slight adjustments to the pretreatment protocol between cases.

4.2.3.2.3 NICK Columns

The use of nick columns is an additional step in some nick translation protocols (Syrjänen, 1992) to absorb unincorporated label that could result in increased background. However in the protocol followed in this study, there was a reduction in signal intensity, therefore this step was not incorporated into the methodology (Figures 19C and D). This may have been due to a DNA precipitation step following the nick column protocol, as either nick columns or DNA precipitation are usually recommended for the removal of unincorporated nucleotides.

4.2.3.2.4 Suppression Hybridisation

COT-1 DNA is human DNA enriched for repetitive sequences (Gosden & Hanratty, 1991). The addition of COT-1 DNA, known as suppression hybridisation (Lichter *et al*, 1990) blocks repetitive sequences and is essential in the hybridisation of unique sequences for avoiding ambiguous signals. If the entire sequence from a cosmid clone is labelled and used as a probe, the interspersed repetitive sequences such as *Alu* and *Kpn* elements may confound the results (Nisson & Watkins, 1991). In this study the results were improved following the addition of COT-1 DNA (Figure 20). The incorporation of a subtractive hybridisation protocol at the probe production stage to quantitatively remove abundant repetitive sequences such as the *Alu* elements (Davidson *et al*, 1998) is an alternative but somewhat complex alternative to the simple addition of

COT-1 DNA when the nick translation products are precipitated. In this study, COT-1 DNA was precipitated with the cosmid DNA which gave acceptable results, that did not warrant further investigation.

4.2.3.2.5 Denaturation

Denaturing of both probe and target DNA renders the double stranded DNA single stranded thus enabling the probe and target to reanneal. Denaturation can be performed by both chemical and physical means. Alkaline denaturation, used in filter hybridisation, may lead to alkaline hydrolysis of the ester linkages that join the label molecule to the nucleic acid (Herrington & McGee, 1992). Most protocols use a combination of heat, salt and formamide, all of which have the ability to break intramolecular hydrogen bonds, thus destabilise hybrids (Mitchell *et al*, 1992). In some situations, probe and target can be denatured simultaneously by diluting the probe in the hybridisation mix, which contains salt and formamide (Herrington & McGee, 1992), applying this to the section; hybridisation can then proceed by lowering the temperature from 72°C to 37°C. But this is not recommended for unique sequence probes into which a competitor such as COT-1 DNA has been incorporated (Gosden & Hanratty, 1991), because the preannealing step at 37°C for 30 minutes is necessary to allow repetitive sequences to reanneal thus excluding them from the hybridisation process.

In this study, the *NAT2* probe was denatured for 5 minutes at 72°C then left for 30 minutes at 37°C (suppression hybridisation, preannealing step), the chromosome 8 probe was denatured for 5 minutes at 72°C and then added to the *NAT2* probe following suppression hybridisation. However, the denaturing step used here has two disadvantages. It is time consuming and there is added exposure to formamide a highly toxic chemical which can cause foetal damage, even though this step was done in a fume hood. Some commercial sources of unique sequence probe *e.g.* the *HER-2/neu* probe (Vysis, UK) require simply the application of the ready diluted probe to the denatured target, and this produces excellent results (Bartlett *et al*, manuscript in preparation). For any future studies involving a unique sequence probe, the denaturation step would be more closely evaluated to ascertain the necessity of separate denaturation and hybridisation. Tissue sections were denatured with 70% formamide, 2 X SSC at 72°C and then probe applied.

background. The previously used 50% formamide, 2 X SSC wash performed at 42°C was compared to 1 X SSC and 2 X SSC washes both performed at 72°C. The latter two have been used, although less frequently than the former with unique sequence probes. The lower temperature wash can be used with the destabilising effect of formamide, which will increase stringency; the high salt concentration washes alone give a higher stringency wash at the higher temperature. For the demonstration of the *NAT2* and alpha satellite 8 probes used in this study, the cleanest preparation was seen with the 1 X SSC wash, with the least precipitate. The 1 X SSC wash is more stringent than 2 X SSC, probably due to the low salt concentration, as high salt concentration washes stabilise hybrids (Mitchell *et al*, 1992). The added advantage of using a high temperature wash is that it does not contain formamide. As already discussed this is a significant hazard. In conclusion, the 1 X SSC wash was the most suitable with these particular probes.

4.2.3.3 Conclusion

Several technical aspects were investigated to ensure the hybridisation was specific and the signal produced unambiguous. Once this aim had been achieved, an assessment firstly of control material then test material was undertaken.

4.2.4 Comparison of FISH with other Molecular Biology Techniques

4.2.4.1 FISH versus ISH

Non-fluorescence *in situ* hybridisation (ISH) can be viewed with standard light microscopy thus tissue morphology is clearly seen, and direct comparison between ISH sections and other histology techniques can be made. This is an advantage over FISH, although with experience, tissue morphology can still be identified as, for example, elastin fibres autofluoresce. In this study the FISH stained sections were compared to sequential haematoxylin & eosin sections. Confirmation of tumour areas was made and regions of differing pathology assessed.

ISH has been widely applied to the study of numerical chromosomal aberrations (*eg* Hopman *et al*, 1991, Poddighe, 1996). Although colour separation is more difficult as chromogens are usually red and brown, dual labelling has been used successfully in conjunction with immunocytochemistry (Speel *et al*, 1994). However, the use of dual and triple band pass filters in FISH enables detailed analyses of chromosomal and genetic aberrations to be carried out not so easily done by ISH. By using a dual labelling FISH technique, with a probe for the centromere of the chromosome which houses the

gene of interest additional information on the genetics of the carcinoma can be obtained. For example, Sauter *et al*, (1995c) using this approach defined amplification of *c-myc* copy number if more than 10% of cells had more *c-myc* signals than chromosome 8. In the study of Wagner and co-workers, (1997) also using dual FISH, they noted that 8p deletions were generally associated with an increased copy number of chromosome 8. These data cannot easily be achieved with ISH and therefore in this study, FISH was used to directly relate chromosome and gene copy number to tumour pathology.

4.2.4.2 FISH versus Flow Cytometry

Flow cytometry allows up to thousands of cells to be scanned per second (Camplejohn, 1992), the advantage is that the ploidy of large numbers of cells can be determined rapidly, compared to the more time-consuming analysis of cells on slides (Cajulis *et al*, 1994). Two parameters can be measured by flow cytometry-the presence of cells with abnormal amounts of DNA, the DNA aneuploid cells, and the S phase fraction, a crude index of cell proliferation (Camplejohn, 1992), and the technique can be used to segregate X and Y sperm.

The disavantage of this technique is that, if solid tumour is being analysed, unless the tumour is microdissected before flow cytometry is carried out, a dilution effect will occur, whereby tumour and normal tissue will be analysed together. In any event, small changes of ploidy are often missed *e.g.* in a slightly aneuploid population and tumour heterogeneity cannot be determined (Ramaekers & Hopman, 1993), due to the limitations of the technique. This is illustrated by the example of a common aberration in TCC, loss of chromosome 9. This is often an early event in the development of TCC and some researchers have suggested that this loss enhances susceptibility to other genomic changes (Ramaekers & Hopman, 1993). If subsequent genetic aberrations involve tetraploidisation, and flow cytometry is applied at that stage in the tumour's development, the chromosome complement will be 4n, but specific chromosomal abnormalities will not easily be detected.

In conclusion flow cytometry is useful in giving an estimate of the total chromosome copy number in a cell (Cajulis, *et al*, 1994) but subtle genetic changes could be missed and these may be more profound in the analysis of tissue sections.

4.2.4.3 FISH and Comparative Genomic Hybridisation

Comparative genomic hybridisation (CGH) (Kallioniemi *et al*, 1995) has recently been developed to screen for chromosomal losses and gains. The method involves the comparative hybridisation of differentially labelled tumour and normal DNA to normal

metaphase cells. This gives an advantage over methods such as FISH, which can only assess two or three chromosomes or genes *in situ* at one time, whereby the whole genome can be screened in one experiment (Mahdy *et al*, 1999). This methodology is a hybrid between flow cytometry and *in situ* hybridisation, and highlights genetic abnormalities, which can then be investigated further with *e.g.* FISH. However the technique is again limited if the analysis is performed on tissue sections.

DNA arrays on microchips are the latest technological development for assessing large panels of genes. This technology has been successfully applied to the simultaneous expression of thousands of genes and to large scale gene discovery (Ramsay, 1998). As with CGH the ability to screen many genes or chromosomes simultaneously is of enormous benefit, although at present DNA arrays on microchips can only be done on blood or lymphocyte preparations. Spectral karyotyping (SKY) combines the sensitivity and specificity of FISH with the global screening ability of conventional cytogenetics (Mark, 1998). This is another example of a technique that enables a high throughput of gene screening, but as with CGH and DNA arrays, the disadvantage is that expensive instrumentation is required, outwith the budget of most teaching hospitals and universities.

In conclusion, CGH and DNA arrays are very powerful tools in the screening of tumours for gross genetic changes, but subtle genetic aberrations may be missed especially if whole tissue sections are utilised. For example, CGH will not detect tumour suppressor gene inactivation that does not involve physical loss *e.g.* mitotic recombination and similarly activation of oncogenes by any other means than amplification is not detected (Kallioniemi *et al*, 1995).

4.2.4.4 Other Molecular Biology Techniques and FISH

RFLP (restriction fragment length polymorphism) analysis utilises specific restriction enzymes to study loss of heterozygosity (Yasui *et al*, 1992). This has been used in genotype analyses, for example to identify specific gene deletions (Risch *et al*, 1995). A comparison of *in situ* hybridisation and RFLP analysis by Poddighe *et al*, (1996) acknowledged that RFLP analysis gave valuable information on specific allelic losses, that was not possible using *in situ* hybridisation. However, the detection of genetic aberrations on a cell by cell basis was only possible using the *in situ* approach.

Techniques such as Southern blotting give an overview of changes that dominate a tumour population (Poddighe *et al*, 1996) and indeed Southern blotting and polymorphic microsatellite analysis have been used to provide information about the

genetic alterations which underly the development of solid tumours (Murphy *et al*, 1995). But the overriding disadvantages with Southern blotting and RFLP analysis are that a dilution effect will occur, as whole tissue sections are used in the analysis. Thus there will be an admix of tumour and normal tissue, subsequently no information can be obtained on tumour heterogeneity, nor can tumour be directly compared to pathologically normal tissue.

4.2.4.5 Conclusion

None of the above techniques when compared to FISH can be used *in situ*. Although in theory they can all be applied to routinely formalin fixed and paraffin processed tissue if microdissection of tumour areas is initially carried out, the quality of the DNA is fragmented and may not be easily analysed. In addition, FISH analysis also avoids the problem of homozygosity encountered in LOH studies such as RFLP. FISH remains a powerful tool for studying genetic anomalies and will have a place in the molecular biology laboratory for some time to come.

The genetic aberrations most commonly associated with TCC, which have been initially characterised with CGH (Kallioniemi *et al*, 1995) and RFLP (Risch *et al*, 1995) can be further assessed with FISH and specific probes (Mahdy *et al*, 1999). Using an *in situ* technique on stored material from the pathology archives, genetic aberrations can be related to clinical outcome. As clinical material was used throughout the study, FISH was the most suitable technique to use in this study.

4.3 Analysis of Results

4.3.1 Range of Tissue Used for Analysis

The assessment of tumour ploidy *in situ* is relatively recent (Hopman *et al*, 1988). The advantages over flow cytometry, the existing technique for measurement of ploidy in interphase nuclei have already been discussed (Section 4.3.3.2).

Initial studies were made using metaphase spreads, using cells from a bladder cancer cell line and whole nuclei isolated from thick (50 micron) sections which were resuspended and dropped onto slides (Hopman *et al*, 1988). There are essentially two types of preparation that have been used to analyse the results of *in situ* hybridisation experiments: cells derived from cytology specimens or from paraffin wax tissue blocks or as intact sections from tissue imprints and frozen or paraffin wax embedded tissue blocks. The advantages and disadvantages of each type of methodology are discussed below.

4.3.1.1 Cytology

Bladder washings and urine samples are the simplest way of obtaining material for DNA analysis. However, urine is often acellular and DNA analysis cannot subsequently be performed. Bladder washings increase the cellular yield; FISH as applied to bladder washings was comparable to the detection of malignant cells by cytology with the advantage that the specimen was more cellular in terms of sensitivity and specificity (Cajulis *et al*, 1994). However this type of specimen may contain both tumour and normal cellular components.

4.3.1.2 Isolation of Nuclei

The dissociation of nuclei from 50 micron tissue sections cut from paraffin wax tissue blocks has been used to analyse several common genetic aberrations in bladder cancer (Hopman *et al*, 1988, 1991, Sauter *et al,* 1995 a-d). The single cell suspensions were dropped onto glass slides for further analysis. Although nuclear truncation in thin sections is avoided, the relationship between neoplastic and non-neoplastic tissue and correlation with pathology in haematoxylin and eosin sections is not possible. Two other disadvantages are that the resultant cell suspension is an admix of tumour and normal cells, which may be considerably more difficult to discriminate between than if assessment was performed *in situ*. In addition the analysis of a small biopsy may not be possible due to the limited material available. There is another disadvantage with this

methodology as it incorporates another step in the FISH technique, which may prolong the protocol by a day.

However, in both methods outlined above, large numbers of nuclei would have to be assessed to be confident that any genetic abnormalities had not been missed. It may be difficult to differentiate tumour from normal or inflammatory tissue components. This also impinges on the methodology for assessing the preparations, further discussed in Sections 4.3.2 and 4.3.3.

4.3.1.3 Tumour Imprints

The use of tumour imprints from frozen or freshly excised tumour again enables whole nuclei to be analysed. However, the disadvantage is that tumour cells may have differing characteristics from normal cells. They may more readily detach from the tumour due to the increased production of degradation proteins. If frozen material is available, parallel studies using FISH, flow cytometry and comparative genetic hybridisation are more easily facilitated and direct comparisons of each technique can be made (Mahdy *et al*, 1999). All these studies have contributed to our knowledge of the genetics of bladder cancer. However, the practicalities in a clinical setting of obtaining fresh material become limited. If there is not a dedicated surgeon in the operating theatre, all tissue will follow the routine procedure, whereby tissue is placed in neutral buffered formalin for processing in the pathology laboratory. Assessment of the sections would follow the same protocol as for cytology specimens.

4.3.1.4 *In situ* Analysis of Frozen and Archival Tissue

Frozen sections are probably the most suitable type of preparation for assessing chromosome or gene copy number *in situ* as tissue will not have been placed in fixatives which can damage DNA (Williams *et al*, 1999). The limitation of frozen material is that long term storage may be problematic and the material is less easy to handle than formalin fixed and paraffin wax processed tissue. Stored archival material *i.e.* formalin fixed and paraffin wax processed tissue, is the most accessible, in terms of using tissue for further study. However, technically it is more difficult to work with archival tissue than cell spreads or imprints. Optimisation of the proteolytic digestion step is a prerequisite of the methodology. As discussed in Section 3.2.2, a compromise between optimal digestion and preservation of tissue architecture must be achieved.

The use of archival material, as thin sections, provides the greatest amount of information on ploidy using the technique of FISH, in a diagnostic setting. However, as outlined in more detail in Section 4.3.2, assessment of the sections requires careful

evaluation, due to nuclear truncation artefact and there also needs to be strict quality control to ensure that accurate assessment of ploidy is achieved.

4.3.2 Evaluation Criteria

Hopman *et al*, (1988), used non-fluorescence *in situ* hybridisation (ISH) on interphase nuclei. These researchers outlined criteria for evaluation including avoidance of nuclear overlap, which would result in overestimation of the true copy number. The analysis of probe signal involved scoring spots of equal intensity, avoiding minor hybridisation signals, and if split spots (representing chromosomes about to enter "S" phase) very close together were seen again scoring these as one. All these criteria will bypass overestimation of copy number. Many researchers have produced papers using ISH and FISH since then, some referring to the criteria of Hopman *et al*, (1988), (for example, Cajulis *et al*, 1994). Others (*e.g* Sauter *et al*, 1995b and Pycha *et al*, 1997) refer to previous papers such as that of Waldman *et al*, (1991). The material used by all these researchers was whole nuclei, either as touch preparations (Waldman *et al*, 1991), barbotage specimens (Pycha *et al*, 1997) or dissociated nuclei (Hopman *et al*, 1988).

Once the definition of a true hybridisation signal is made, the number of nuclei to be assessed per tumour section must be determined. Two hundred to 500 nuclei were scored for each hybridisation and damaged and overlapping nuclei were ignored (Waldman *et al*, 1991). Variability remains in the number of nuclei assessed and in some studies the number is not specified (Misra *et al*, 1995). The exact number of nuclei that should be assessed per specimen will vary with the size of tumour.

Guidelines for accurate quantitation of copy number may include: initial evaluation of the whole tissue section, with reference to the haematoxylin and eosin stained tissue section, to identify tumour areas and avoid missing areas with different tumour pathology; distinct areas should be analysed and a minimum of 200 nuclei should give an accurate measure of copy number in a small biopsy. Larger tumours will require additional analysis of more nuclei to be representative of the specimen as a whole from different areas within the section. Previous studies within this laboratory (Bartlett *et al*, 1998, Watters *et al*, 1999) have shown that analysis of 200 to 600 nuclei (depending on section size) gives an accurate measure of copy number. Too few nuclei may miss a lesion but too many results in unnecessary analysis.

4.3.3 Definition of Aneusomy

As discussed in Section 4.3.1, there are essentially two different types of cell preparations that have been used in the analysis of *in situ experiments*, either dissociated nuclei or intact tissue preparations, and the former preparation has been more widely used.

Assessment of chromosome copy number in dissociated nuclei, reflected the major (>50%) or dominant (20-50%) population present, if no aneusomic (*i.e.* other than 2 copies/nucleus) subpopulation was present at >50% of the total, then the largest aneusomic population having >20% of total cells was used (Waldman *et al*, 1991). Thus a tumour was considered disomic if no other subpopulations had >20% of total cells. This is a cumbersome method of analysis and a simplified method would be preferable.

Variation in the cut off for aneusomy, from 5% (Cajulis *et al*, 1994) to 10% (Mahdy *et al*, 1999) to 20% (Waldman *et al*, 1991) has also been reported. Thus if between 5 and 20 nuclei per 100 were aneusomic this would give a diagnosis of aneusomy, depending on the study. The weakness with this type of analysis is that these preparations contain a mix of normal and abnormal cellular elements, and it may be necessary to assess many nuclei before an accurate genetic profile is achieved. As Figure 13 illustrates, an area is easily identified for analysis with an intact tissue section, but if the tissue section had been dissociated and analysed, at least 80% would be non-tumour and may give a false result.

In previous studies of assessment of intact tissue sections, such as Pauletti and co-workers, (1996), 200 to 300 signals in randomly selected tumour nuclei were counted, less in cases of obvious gene amplification. Where the tumour can be identified unambiguously, assessment of as few as 100 or even 60 nuclei may be acceptable for an accurate genetic profile. In this study, two simple methods for assessment of aneusomy were developed. The first based on previous research (Bartlett *et al*, 1998, Watters *et al*, 2000) involved the summation of all signals observed then divided by the number of nuclei in a particular field, the mean chromosomal copy number (MCCN) or the mean gene copy number (MGCN). Established values reflecting normosomy were from 200 nuclei for both chromosome 8 and *NAT2* over 10 control sections. Thus figures for loss or gain were defined taking into account the mean control values $\pm$ 3 X SD *i.e.*99% confidence interval (Table 8). This was a stringent cut-off, but gave a robust method for accurate detection of genetic and chromosomal aberrations *in*

situ in thin tissue sections. By following this method, confidence in the assessment of tumour sections in this study was afforded. In addition at least 200 nuclei were counted, from one area, and signals in up to 3 areas were counted where the material was plentiful *i.e.* to 600 nuclei were assessed.

Alternatively the mean *NAT2:8* ratio was established and loss of *NAT2* relative to chromosome 8 was defined. This was useful to detect losses of gene copy number, but information on polysomy or monosomy could not be derived. It was therefore necessary to take into account the copy number of chromosome and gene (Misra *et al*, 1995, Pauletti *et al,* 1996). The ratio of gene to chromosome copy number is also frequently used, (*e.g.* Pauletti *et al*, 1996).

In conclusion, unless the signals in a large number of nuclei in dissociated material are counted, the copy number may not be representative of the specimen being analysed. Assessing copy number in intact tissue sections avoids this problem, but the use of thin tissue sections requires analysis of normal copy number in several tissue sections to encompass variability in nuclear slicing.

4.3.4 Control Material

In this study, several tissue sections normosomic for chromosome 8 and *NAT2* were assessed before the tumour sections were analysed, and normosomic bladder tissue as controls were incorporated into each FISH run. Previously, researchers have reported using normal lymphocytes as controls for disomy (Sauter *et al*, a-d, 1995, Murphy *et al*, 1995) or normal parenchyma from tumours to determine the background levels of trisomy and tetrasomy, (Corless *et al*, 1996). Correlation with other molecular biology methods was used to control for FISH reproducibility (Misra *et al*, 1995) but no direct reference to controls was made. The salient point in the use of control tissue is that a similar type should be used as the test. In that way conditions of pretreatment and hybridisation of material are standardised and a control is for the specificity of probe hybridisation only. This approach was used throughout this study and was a useful measure of technical reproducibility.

4.3.5 Quality Control

Eighty per cent of controls, to establish normal values, were dual scored, by observers from two different laboratories, as were 31% of tumours. This approach has previously been taken in this laboratory, (Bartlett *et al*, 1998, Watters *et al*, 2000). Good

concordance of results meant that inter observer variation was established at ≤12%, which reflects a robust and reproducible technique.

4.3.6 Conclusion

In order for the molecular biology technique FISH to become part of a diagnostic service, clear and simple guidelines for assessment must be laid down, which must be unambiguous and reliable. The described method for analysis of the results in this study falls into these criteria and encompass the shortcomings of other methods previously reported.

4.4 Evaluation of TCCs

4.4.1 Range of Abnormalities Detected

Twenty of 37 TCCs were abnormal. Distinct patterns of abnormality were observed: normosomy 8 and loss of *NAT2*, polysomy 8 and loss of *NAT2* and polysomy 8 with retention of *NAT2* copy number. These differing abnormalities are discussed in more detail as follows.

4.4.1.1 Normosomy 8 & Loss of *NAT2*

One TCC (from a non-recurrent patient) had this abnormality. This implies a loss of genes in the region 8p22, probably with loss of function of the retained copy indicative of a tumour suppressor gene (TSG) locus, which may play a role in the development of TCC (Knowles *et al*, 1993). Loss of heterozygosity (LOH) on 8p is frequent in many types of solid tumours, such as prostate, breast and bladder (Takle & Knowles, 1996). Several genes have been identified which could potentially act as tumour suppressor gene. The region of deletion at 8p21.3-p22 contains the *MSR* gene. Homozygous deletion of *MSR* (macrophage scavenger receptor gene) mapped to 8p22 (MacGrogan *et al*, 1996) has been described in a metastatic prostate cancer sample (Takle & Knowles, 1996). The genes for the metabolic enzymes N-acetyltransferase (*NAT1* and *NAT2*) have been mapped to 8p22 (Matas *et al*, 1997). The enzymes function to acetylate potential carcinogens. The increased incidence of slow acetylators who develop bladder cancer following occupational or smoking exposure (Cartwright *et al*, 1982, Risch *et al*, 1995) implies a loss of function of one of the alleles at this locus. However either *MSR* or *NAT1/NAT2* are unlikely to be candidate tumour suppressor genes because of their specific functions that do not involve cell cycle regulation. They

are most likely deleted through a mechanism such as methylation silencing (MacGrogan *et al*, 1996)

A candidate tumour suppressor gene is homologous to an extracellular domain of the PDGF (platelet derived growth factor) receptor gene mapped to the region 8p21.3-22. PDGF-B is identical to the proto-oncogene protein Sis and controls the 'restriction point' in the cell cycle, at the G0 or G1/S transition (PDGF Home Page). Structural rearrangements and mutations have been found in a small number of tumours of various types, although at lower frequency than would be expected on the known frequencies of 8p LOH (Takle & Knowles, 1996).

Using 19 microsatellite markers on 8p, Ohagki, and co-workers, (1999) describe a common region of deletion which overlaps and narrows the 8p21.1-pter deletion described by Takle & Knowles (1996) in bladder cancer. Inactivation of a tumour suppressor gene (s) at 8p22 is postulated to play an important carcinogenic role in prostate and other cancers including that of the urinary bladder, although the precise gene(s) have not been identified (Ohagki *et al*, 1999).

4.4.1.2 Polysomy 8 and Loss of *NAT2*

In the TCCs from two patients this abnormality was observed.This aberration suggests the formation of an isochromosome 8q, a common finding in many tumours including the bladder cancer cell line, RT112 (Stacey *et al*, 1999), whereby simultaneous 8q gain occurs with 8p loss (Wagner *et al*, 1997). There may also be LOH occurring simultaneously as reports of 8p deletions and 8q gains are a frequent aberration in other tumours (Wagner *et al*, 1997). There has also been a suggestion that if a loss of 8p occurs, this then confers a selective advantage for whole chromosomal gain to occur in the following cell divisions (Sato *et al*, 1999).

4.4.1.3 Polysomy 8 with Retention of *NAT2* Copy Number

The TCCs from ten patients had this abnormality. This implies duplication of chromosome 8 without major changes in *NAT2*, but in other regions on chromosome 8, such as amplification of *c-myc* or at the region 8p12 (Wagner *et al*, 1997). Trisomy and polysomy 8 are associated with other carcinomas, including those of ovary, prostate and breast (Mark *et al*, 1997). Trisomy 8 was associated with higher stage disease in breast carcinoma and a gain of chromosome 8 was associated with systemic progression in prostate cancer (reviewed by Mark, *et al*, 1997). Chromosome 8 aberrations (losses and gains) are frequently observed in ovarian cancer, where it may be the lone abnormality, suggesting a role for abnormal chromosome 8 in carcinogenesis (Wolf *et al*, 1996).

Aneusomy 8 is associated with an aggressive phenotype in bladder cancer (Knowles, *et al* 1993, Sauter *et al*, 1995c, Wagner *et al*, 1997), and in this study was more commonly observed in patients with muscle-invasive disease. This suggests that polysomy of chromosome 8, as observed in this study, is a later event in bladder carcinogenesis, possibly a marker of disease progression.

The oncogenes that might drive the process of polysomy in the absence of significant alteration of *NAT2* copy number include *c-myc* at 8q24, and an as yet unidentified oncogene at 8q12 both of which were associated with bladder cancer progression (Wagner *et al*, 1997).

4.4.1.4 Increase in Copy Number of Chromosome 8 and *NAT2*

Seven patients' TCCs had this abnormality. This suggests that whole chromosomal duplication has occurred. Aberrations of chromosome 8 are common in many carcinomas and regions on both chromosome arms have been extensively mapped. Although the retention of *NAT2* implies that the region 8p22 has not been lost, a deletion telomeric to this region may have occurred as 8p23.2 to 8p22 is thought to contain a tumour suppressor gene (Eydmann & Knowles, 1997).

4.4.1.5 Conclusion

Abnormalities of chromosome 8 and/or *NAT2* were common in this group of tumours, although no losses of chromosome 8 copy number were observed. Complete chromosomal loss of 8 does not appear to be an aberration seen in epithelial derived tumours (Knowles *et al*, 1993, Sauter *et al*, 1995c).

4.4.2 Association of Stage & Grade with Genetic and Chromosomal Aberrations

4.4.2.1 Polysomy 8

Nineteen of 37 tumours (51%) had polysomy 8 and this was associated with advanced stage ($p=0.024$) and grade ($p=0.0056$). Although the numbers are smaller, the results are similar to those of Sauter *et al* (1995c) who detected polysomy 8 in 41 of 87 (47%) of bladder cancer cases, and this aberration was strongly associated with high stage and grade. Aneusomy is associated with a poorer prognosis as an accumulation of chromosomal aberrations leads to increased instability of the genome (Ried *et al*, 1999). Genes have been identified on chromosome 8 that may contribute to carcinogenesis and progression such as the oncogene *myc* located at 8q24 which was overexpressed in

bladder tumours and associated with low grade bladder cancer (Sauter *et al*, 1995c). Gains at 8q24 were associated with higher stage bladder carcinomas by Wagner and co-workers, (1997) by FISH which contradicts a previous study (Sauter *et al*, 1995c) by immunocytochemistry. The protein product of the *myc* oncogene when overexpressed, has been hypothesised to cause degradation of $p27^{kip1}$ leading to activation of cyclinE/cyclin dependent kinase 2 and cell proliferation (Sato *et al*, 1999). The amplification reported at 8p12 has led to suggestions of a candidate oncogene on 8p, the heregulin gene coding for a ligand for the *erbB2* oncogene and fibroblast factor 1 (Wagner *et al* 1997).

In this study, the association of polysomy 8 with high stage and grade reflects previous research (Wagner *et al*, 1997 and Sato *et al*, 1999). Although there only appears to be two candidate regions for oncogenes, amplification at 8p24 and 8p12 (Wagner *et al*, 1997), 8p contains at least three regions of deletion, and at least two candidate tumour suppressor genes (Takle & Knowles, 1996) all of which have the potential to exacerbate the propensity of a cell to become polysomic. Intriguingly, the region 8p12 has been reported as amplified (Wagner *et al*, 1997) as well as deleted (Eydmann & Knowles, 1997), which may further confound the exact mechanisms whereby oncogenesis occurs.

In malignant cells, functional gain or loss of oncogenes and tumour suppressor genes is often mirrored by the acquisition of chromosomal aberrations (Ried *et al*, 1999). Thus, from previous studies, there would appear to be a confounding effect of polysomy possibly due to simultaneous loss of one region on chromosome 8 and gain of another, contributing to the increased stage and grade of tumours associated with this aberration, reflecting the results of this study.

4.4.2.2 Abnormalities of *NAT2*

Grade was associated with any abnormality of *NAT2* (p=0.006) but not with stage in this study. A closer evaluation of the results, demonstrated that the pTa carcinomas had all lost the *NAT2* gene whereas the pT1 and pT2+ retained the gene with similar copy number to chromosome 8. The grade 1 and 2 carcinomas had lost *NAT2*, the grade 3's had retained *NAT2*.

Three different putative tumour suppressor regions on 8p have been localised to 8p21.3-22 (Takle & Knowles, 1996) and at 8p23.2-22 (Eydmann & Knowles, 1997). The region 8p22 is a common region of deletion in many cancers including hepatic, colorectal and lung (Oghaki *et al*, 1999). The genes encoding the *N*-acetyltransferases

(*NAT1* and *NAT2*) have been localised to 8p22 (Matas *et al*, 1997). Loss of heterozygosity (LOH) at 8p22 that encompasses the functional *NAT* alleles will result in loss of function of these enzymes (Matas *et al*, 1997). Prostatic primary carcinomas show LOH at 8p22 in their metastases (Oghaki *et al*, 1999). There has been a suggestion that the *NAT2* genes are a marker for loss (Thygesen *et al*, 1999) as no other expressed sequences are known in this region.

NAT1 lies very close to *NAT2* (Matas *et al*, 1997) in the region 8p22 and there is a strong possibility that not just *NAT2* but both *NAT1* and *NAT2* genes will have been lost or retained (Thygesen *et al*, 1999). Where loss has occurred if both genes are involved, this may confer a selective advantage for bladder carcinomas to progress, as the functions of both NAT1 and 2 will have been lost. Although a low number of carcinomas showed loss of *NAT2*, 3 of 5 were in patients that had muscle-invasive disease at some point in their clinical history. *NAT1* and *NAT2* are polymorphic genes and vary in their ability to metabolise aromatic amines, potent carcinogens, as discussed in the introductory chapter and when lost may in part be responsible for the malignant potential of bladder cells. Immunohistochemical studies (Stanley *et al*, 1996) have demonstrated, using polyclonal antisera to NAT1 and NAT2, that NAT1 but not NAT2 is expressed in the bladder and a diminution in NAT1 was detected in highly invasive tumours. There is thus a strong possibility that both enzymes are dysfunctional in the cases reported here and that this confers an increase in the potential for aromatic amines to be activated rather than detoxified.

4.4.2.3 Abnormalities of Chromosome 8 and *NAT2*

Fifty four percent (20/37) bladder carcinomas had abnormalities of either chromosome 8 or *NAT2* or both chromosome and gene, and these were associated with high stage ($p=0.03$) and grade ($p=0.01$). The loss of *NAT2* was more frequently seen in lower stage and grade carcinomas as discussed above, whereas a high copy number was observed with higher stage and grade. Sato and co-workers (1999) hypothesise in prostate cancer that accumulation of genetic aberrations occurs in 3 steps, first mutation or loss at 8p22, then whole chromosomal gain of chromosome 8, perhaps on the chromosome with a loss of 8p22 and thirdly 8q is gained, possibly as a result of isochromosome formation at 8q. Alternatively, the oncogene *myc* (at 8q24) may drive the process of progression. In prostatic carcinoma, *myc* amplification was associated with poor prognosis, independently of other prognostic factors (Sato *et al*, 1999). From the results of this study, it would appear that loss of *NAT2* occurs earlier than other

aberrations in the development of bladder cancer thus supporting a previous report (Sato *et al*, 1999).

The gains observed in this study may represent whole chromosomal gain as they were always seen in tumours with polysomy 8, and the gene copy number reflected that of the chromosome. Two examples of tumours with similar *NAT2* copy number to chromosome 8 are shown in Figure 21 D and E. These were observed in invasive (stage pT2 or above) tumours. This aberration, concomitant polysomy 8 with almost equivalent copy number of *NAT2*, was also reported by Stacey and co-workers (1999) using FISH to analyse copy number. These aberrations were more common in higher stage (pT1 and pT2) tumours.

4.4.3 Aneusomy and Outcome

The NR patient with polysomy 8 was staged and graded as pT1G3, the remaining patients with polysomy 8 were muscle-invasive at presentation or developed this pathology. Two exceptions were the primary tumour from a patient (pTaG1) who had several recurrent non-invasive tumours, although this tumour did exhibit heterogeneity, known to be associated with an inherent genomic instability (Yokogi *et al*, 1996). This patient was normosomic for all subsequent events studied; one recurrent tumour from another patient in the RNP category was aneusomic for chromosome 8, although the copy number was at the low end of abnormal, with a mean copy number of 1.75.

Patients were followed through recurrence to progression and the copy number was compared to outcome. When assessing chromosome 8 or *NAT2* by mean copy number, 8/9 (89%) of patients with muscle-invasive disease at presentation or who developed this pathology had aberrant gene and chromosome copy number, whereas only 4/10 (40%) of patients with non-invasive disease also had aberrations. There is an indication form these results that polysomy 8 may be a marker of progression. Sauter *et al* (1995c) observed that tumour cell proliferation, gain of *c-myc* copy number and polysomy of 7, 8 and 17 were strongly associated with a more aggressive phenotype, in his series of 87 tumours. The observation that patients with abnormal chromosome and/or gene copy number in their primary TCCs were predominantly from the group that progressed to muscle invasive disease (or had that phenotype at presentation) implies that these genetic aberrations are associated with an aggressive phenotype. This is

largely in accordance with previous studies, (for example Knowles *et al*, 1993, Sauter *et al*, 1995c, Wagner *et al*, 1997).

4.4.4 Association with Other Chromosome Abnormalities

Previously alpha centromeric FISH probes for chromosomes commonly aberrant in TCC namely 7, 9, 10, 11 and 17 have been used to investigate abnormalities in some of the patients recruited into this study. Apart from the non-recurrer with normosomy of 8 and loss of *NAT2* (NR4) all the patients with abnormal 8/*NAT2* copy number had abnormalities of at least one other chromosome (Table 21). This is consistent with the genetic model for cancer progression discussed in Chapter I, whereby an accumulation of genetic aberrations is required to fully acquire a malignant phenotype. The high prevalence of abnormalities in patients in the RP and PP categories fits with the model proposed by Reznikoff and co-workers (1995), (Figure 6), whereby aberrations of chromosome 8 are associated with the later stages of bladder cancer progression.

4.4.5 Tumour Heterogeneity

Tumour heterogeneity was noted in 16.2% (6/37) of patient's TCC events. This is a particular strength of an *in situ* technique, where different areas in one tissue section can be analysed, and as previously discussed, this characteristic cannot be detected in other molecular biology techniques such as flow cytometry and RFLP analysis. Of the six heterogeneous TCC's, five were from patients with either muscle-invasive TCC at presentation or who subsequently developed this phenotype. Sauter *et al*, (1995d) demonstrated a high degree of aneusomy with heterogeneity and suggest that heterogeneity is a hallmark of genomic instability and that very heterogeneous tumours may represent an aggressive sub-type of bladder carcinoma. Yokogi *et al* (1996) also studied heterogeneity in bladder tumours and hypothesise that tumour heterogeneity could be a potential marker of progression as it was associated with a more aggressive phenotype.

4.4.6 Exposure to Carcinogenic Chemicals and Genetic Aberrations

Smoking is associated with slow *NAT2* genotype, and 12/13 patients with smoking history in this study were smokers, associated with an increase risk of developing bladder cancer (Morrison *et al*, 1984, Sørlie *et al*, 1998). Risch *et al*, (1995),

compared smokers versus non-smokers in the control and bladder cancer patients, and showed that nearly twice as many patients with bladder cancer and slow *NAT2* genotype were smokers compared to non-smokers (Figure 9). In this study smokers were not associated with any particular clinical outcome, although previously an association with a more aggressive clinical course for bladder cancer has been made in smokers (reviewed by Landman & Droller. 1998). But this may be due to the small numbers included in this study.

No conclusion could be reached regarding occupational exposure and genetic aberrations as only one patient (a foreman painter) was in a high risk occupation for developing bladder cancer. This may reflect the geography of the region from which this patient cohort was selected, as industries such as dye factories have not been present, therefore there would be a smaller number of occupationally exposed bladder cancer patients as compared for example to previous research (Cartwright *et al*, 1982).

4.4.7 Conclusion

In the context of this study the results with FISH on archival sections of bladder carcinomas exemplifies the applicability of this approach to a diagnostic setting. No special procedure was involved in order for the fluorescence *in situ* hybridisation to be performed. The material was easily accessible from the pathology archives and as demonstrated even in this small patient cohort (n=19), a large amount of data was generated. This was related to pathological staging and grading and clinical disease course. Additionally, pathology could be directly related to FISH by carrying out the FISH *in situ*.

This study looked at the copy number of chromosome 8 and *NAT2* in patients with clinical histories from those with non-recurrence following the primary TCC through to patients that recurred but did not develop muscle-invasive disease through to patients that did develop muscle-invasion and finally to those with muscle-invasion at presentation. A higher proportion of patients in the latter two categories had abnormal chromosome and/or gene copy number compared to those in the first two categories. Data from chromosome 8 in this study showed an association with polysomy and progression, the highest proportion of patients with this aberration had muscle-invasive disease. The high proportion of patients with aberrations of 8 and *NAT2* (89%) with muscle invasion at presentation or who developed this aggressive phenotype strongly

contrasts with only 40% from the two groups with non-invasive phenotype. This is in concordance with previous studies that have shown that deletions of 8p are more common in more advanced bladder cancer (Knowles *et al*, 1993, Wagner *et al*, 1997). The data from *NAT2* also reflects the high proportion of LOH of 8p previously observed in patients with bladder cancer (Knowles *et al*, 1993).

The results of this study demonstrate that by studying the genetics of a disease entity, in this case TCC, by following a patient's disease course over several years a substantial amount of clinically relevant data can be gathered. But it must be borne in mind that any test that challenges existing diagnostic methods, even as an adjunct, requires to be accurate and reproducible. Careful attention to quality control is essential and inter laboratory tests would need to be standardised. It would also be necessary to reproduce the results with large statistically significant numbers. However, in the future, for example, a radical cystectomy could be offered to a patient whose genetic profile on his primary carcinoma predicted progression, and we may not be many years away from a third arm to the diagnosis of TCC, in the form of molecular diagnostics.

An interesting review of genomic changes associated with progression from benign conditions such as a colorectal adenoma to adenocarcinoma was recently published (Ried *et al*, 1999). They brought together studies using comparative genomic hybridisation (CGH) to identify aberrations and associate them with tumour progression. This consolidates previous studies that have linked increasing aberrations with higher stage, more aggressive carcinomas and consequent poor prognosis. Specific aberrations are associated with initiation, such as loss of chromosome 9 in bladder cancer (Knowles, 1995) and with progression such as gain of chromosome 13 in colorectal cancer (Ried *et al*, 1999). The specific changes associated with particular neoplasms warrant their translational application into diagnostics (Ried *et al*, 1999).

With regard to the results of this study, various aspects could be further investigated. The first is the link with polysomy 8 and progression. This could be done by recruiting more patients with complete follow-up, in the progressed groups and performing FISH with a probe for chromosome 8, possibly extending this to include a probe for 8q24. With respect to *NAT2*, it would be interesting to compare the results with *NAT1*, and a probe has now been described (Stacey *et al*, 1999).

The region 8p is undoubtedly of interest in the progression of bladder cancer. Researchers such as Eydmann & Knowles, (1997) Oghaki and co-workers (1999) in

mapping studies of chromosome 8 have identified areas that may contain tumour suppressor genes. This laboratory is currently recruiting more patients to expand the research into a link with progression and polysomy 8, with a probe for *c-myc*. Fine mapping of the 8p12 region may begin to elucidate the area where the putative tumour suppressor lies.

In conclusion, the FISH technique is especially robust in a clinical setting and the presence or absence of specific genetic anomalies has the potential to predict the malignant potential of a carcinoma. In this context the emergence of molecular pathology as an additional diagnostic marker, together with clinical and pathological diagnoses becomes a realistic goal in patient management. Ultimately the contribution of a genetic profile can lead to refinement of treatments and improvement in survival.

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Appendix I: Patient Data

The table on the folowing page contains data obtained from patient notes, incorporated into the patient data

CATEGORY	NAME	DOB	Occupation	Diag	Died	last seen	follow-up (months)		cause of death	age(years
NR	GR	26/04/37	labourer	17/06/92		01/06/94	24			55
NR	JS	09/02/10	steel metal worker	13/07/87	15/04/96		106	DIED	bronchopneumonia	77
NR	RS	03/03/10	production manager	28/03/88	18/04/96		97	DIED	pneumonia	78
NR	AP	14/11/24	ret chief const	01/02/86		14/06/94	101			62
NR	GC	02/03/40	engineer	18/06/91		31/05/94	35			51
NR	AM	01/09/22		17/10/90		02/11/95	61			68
RNP	FM	09/08/32	foreman painter	07/07/83		22/09/94	135			51
RNP	JS	16/08/16		10/04/84	26/01/88	25/08/87	45	DIED	ca hypopharynx	68
RNP	AF	17/03/29		20/06/86		20/12/87	18			58
RNP	MC	24/04/02		18/02/86	12/02/89	19/01/85	36	DIED	IHD/pneumonia	82
RP	WB	11/05/38		08/09/81	29/05/94		152	DIED	inv. bl. Ca	43
RP	JM	06/04/35		19/06/91		31/08/92	14			55
RP	TD	19/12/02		16/11/82	03/04/86		41	DIED	met.bl.ca	80
RP	DM	20/08/32		10/07/93		10/04/96	33			61
PP	BM	19/03/31		22/01/87		04/03/97	122			56
PP	KF	14/07/20		01/08/90	06/05/92		24	DIED		70
PP	JC	31/03/27	housewife	30/01/92		22/07/97	65			65
PP	JM	13/12/21	school janitor	13/05/81		22/12/94	151			60
PP	WW	23/11/31	security guard	31/08/93	27/11/96	01/05/96	33	DIED	met bl ca	62
Abbreviations: patient categories: NR=non-recurrer; RNP=recurrer non-progressor; RP= recurrer progressor; PP= progressed at presentation										
diagnosis; inv bl ca=invasive bladder cancer; met bl ca=metastatic bladder cancer.										

Appendix II: Publications Arising From This Thesis

1. Genetic alterations of n-acetyl transferase in transitional cell carcinoma of the bladder A.D.Watters, M.W. Stacey, J.J.Going, K.M.Grigor, T.G.Cooke, E.Sim, and J.M.S. Bartlett. Presented as a short talk at the British Association for Cancer Research Winter Meeting, 1998.
2. N-acetyl transferase 2 in the recurrence and progression of transitional cell carcinoma of the bladder. A.D.Watters, M.W. Stacey, J.J.Going, K.M.Grigor, T.G.Cooke, E.Sim, and J.M.S. Bartlett. Presented as a poster at the 7th European Workshop on Cytogenetics and Molecular Genetics, 2000.
3. Does Loss of *NAT2* Copy Number Influence Progression in Transitional Cell Carcinoma of the Urinary Bladder? A.D.Watters, M.W. Stacey, J.J.Going, K.M.Grigor, T.G.Cooke, E.Sim, and J.M.S. Bartlett. Submitted to *Carcinogenesis*, 2000.
4. A method for demonstrating the *NAT2* gene by FISH in archival material, manuscript in preparation.