Telomere Function and the Radiosensitivity of Squamous Carcinoma of the Head and Neck

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Submitted in part fulfilment of the requirements of the degree of Doctor of Philosophy.

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November 2004

For Lorna, James and Katherine.

Abstract

Telomeres are tandem repeats of DNA associated with specific proteins. These structures cap eukaryotic chromosomes and have many functions, including maintaining the integrity of the chromosome ends. Telomeres in somatic cells shorten with cell division due to the end replication problem. In the germ line and in embryonic life this is circumvented by the enzyme telomerase, which shows reduced activity in normal somatic cells. Human tumours, including squamous cell carcinoma of the head and neck (SCCHN), must maintain telomere function during very many cell divisions and in most cases this is achieved by the upregulation of the telomerase enzyme. Inhibiting telomerase has been shown to lead to apoptosis in tumour derived cells. Furthermore, as most tumour cells have very short telomeres, they are more likely to succumb to telomerase inhibition than normal cells. This renders telomerase a potential, highly selective anti-cancer target. The telomere is also involved in the repair of DNA double strand breaks, which are the lethal chromosomal lesion produced by exposure of the cell to ionising radiation. Proteins involved in DSB repair are known to be associated with the telomere and telomere dysfunction has been shown to produce radio-sensitivity in mice.

This thesis considers whether the alteration of telomere function by manipulation of the telomerase enzyme can affect the radiosensitivity of *in vivo* derived SCCHN cells and hence whether telomere dysfunction inducing strategies are likely to be synergistic with ionising radiation in the management of SCCHN. The role of telomerase in radioprotecting cancer cells is investigated using the ectopic expression of hTERT in cell lines with high and low levels of telomerase. The effect of inhibiting telomerase expression is examined using a dominant negative telomerase gene. This approach had little success in SCCHN cells and so further experiments designed to elaborate the effect of telomerase inhibition on radiosensitivity were carried out using the small molecule reverse transcriptase inhibitor

3'- azido, 3'- deoxythymidine (AZT). These showed both telomerase suppression and increased radiosensitivity in cells exposed to AZT in culture.

Other suggested factors which may affect the success of radiotherapy for cancer include the missense mutation of the p53 gene. A common polymorphism at codon 72 gives rise to Arginine or proline forms of the protein. This thesis investigates whether this variation affects radiosensitivity in SCCHN cells by assessing a panel of *in vivo* derived SCCHN cell lines.

If the level of telomerase expression does have impact on radiosensitivity, then the use of antitelomerase strategies may be less effective with higher levels of telomerase expression in tumours. Continued selective pressure during tumour progression may mean the emergence of clonal variants with improved telomere function via greater levels of telomerase. This is investigated by anaphase bridge scoring of primary and recurrent archival tumour material. Analysis of cells from primary lesions and then from recurrent disease in the same patient provides information in this regard. This information is of particular relevance since any new therapeutic antitelomerase agent will be administered in phase 1 and phase 2 trials to patients with advanced and/ or recurrent disease.

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Acknowledgements

I would like to thank my supervisor Dr E Ken Parkinson for his unrelenting support and assistance throughout my four year association with his lab.

Dr Paul Shiels, my advisor of studies, has been a positive light throughout and enhanced the value of my research time immeasurably with his advice.

Thank you to NHS Education Scotland, which in its former guise as SCPMDE together with CSO in creating their Clinical Research Fellowship produced a fantastic opportunity, which has never previously existed for a maxillofacial surgeon.

The R9 research group at the BICR have all assisted my research endeavours. Particular thanks to Katrina Gordon for providing BICR7 Tert and BICR7 Neo cell lines used in experiments in chapter 3. Also thank you to June Munro who knows where everything is all of the time in the lab (or knows where you can get it) and to Fay, Hazel, Nighean, Sarah and Vivienne.

Professor Gordon MacDonald provided archival material for chapter 6.

Thanks also to Jorge Curto for statistical advice.

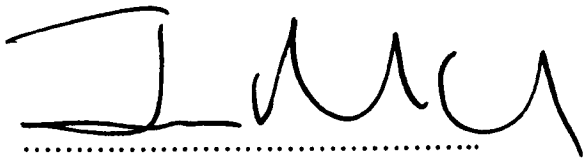
Technical services at the Beatson provided by Peter McHardy and Ian White have been an IT lifeline, performing advanced life support on my (various) laptop computers. I have particularly appreciated the warm "can-do" attitude of the Beatson, brought about by all the staff. Thanks also to Liz in the Library, for tolerance of (very) late returning of items (all of which eventually went back).

Thank you to my clinical colleagues who have supported me during this research time, especially the SpRs.

Most importantly, thank you Lorna, James and Katherine.

Declaration

I am the sole author of this thesis. I have personally consulted all references cited in this work in preparation of the manuscript. Unless otherwise acknowledged, all the work presented in this thesis was performed personally.



James A. McCaul November 2004.

Definitions

ABI Anaphase Bridge Index.

ALT alternative lengthening of telomeres.

AT Ataxia Telangiectasia.

ATCC American Tissue Culture Collection.

ATM Ataxia Telangiectasia Mutated.

AZT 3'-azido, 3'-deoxy thymidine.

BICR Beatson Institute for Cancer Research

BCA Bicinchoninic acid

BSA Bovine serum albumin

CTL Cytotoxic T-lymphocyte

CRUK Cancer Research UK

DMEM Dulbecco's modified Eagles medium

DMSO Dimethylsulphoxide

DNA Deoxyribonucleic acid

DNAPK DNA protein kinase.

DNhTERT The human gene coding for the catalytic component of the telomerase enzyme complex, modified by two amino acid substitutions to produce a non-functioning protein.

DSB Double Strand Break

EDTA Ethylenediaminetetra-acetic acid

EKP Dr E K Parkinson

GM847 An ALT fibroblast cell line

HEPES N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic) acid

HFFs Human foreskin fibroblasts

hTERC The human gene encoding the RNA subunit of the human telomerase enzyme complex

hTERT The human gene encoding the catalytic protein component of the human telomerase enzyme complex.

ISD Information and Statistics Division of the Scottish Executive Health Department.

JMcC Dr J A McCaul

LB Luria Broth

MEFs Mouse embryonic fibroblasts

NES NHS Education Scotland

PAGE Polyacrylamide gel electrophoresis

PCR polymerase chain reaction

PBS Phosphate buffered saline

SF Survival fraction

SCCHN Squamous Carcinoma of the head and neck

SCPMDE Scottish Council for Postgraduate Medical and Dental Education

TE tris, ethylenediaminetetra-acetic acid

TEMED tetramethylenediamine

TIF Telomere dysfunction-induced focus

TPG Telomerase products generated

Tris 2-amino-2-(hydroxymethyl)propane-1,3-diol

TRF1, TRF2 TTAGGG repeat binding factor 1 and 2

TRF Telomere restriction fragment

TRAP Telomere Repeat Amplification protocol.

VEGF Vascular epidermal growth factor.

Wt wild type

Units

^{0}C degree Celsius

bp base pair

Da Dalton

G gramme

G Gravity

Hr hour

Kg kilogram

l litre

μ micro

m	milli
m	metre
M	Molar
Min	minute
Rpm	revolutions per minute
S	second
U	unit
v/v	volume for volume.
w/v	weight for volume

1.Introduction

1.1 Squamous carcinoma of the head and neck

The term squamous carcinoma of the head and neck defines both the tissue of origin and anatomical site of this disease. This is therefore a distinct entity in terms of the cell of origin. In terms of anatomical site, this tumour may arise from the squamous epithelium of the lip, tongue, floor of mouth, nasopharynx, oropharynx, hypopharynx or larynx and still be included within this tumour type. By far the most frequent site involved in this cancer group in Scotland is the epithelium of the oral cavity. (Scottish Cancer Registry 2004)

1.2 Epidemiology and prognosis of squamous carcinoma of the head and neck

The overall 5-year survival for squamous carcinoma of the head and neck (SCCHN) has not altered in more than 30 years. Despite advances in surgical management and radiation and medical oncology the figure of 45 - 50% survival remains static across the developed world. (Jefferies and Foulkes 2001) Worldwide, SCCHN is the fourth most common malignancy in men and the sixth most common malignancy in women, with approximately 500,000 cases being registered in 2000. (Gasco and Crook 2003) In the Scottish context, figures from the Cancer Intelligence Unit of ISD Scotland reveal an incidence of disease which is higher than the UK and is increasing and survival which is poorer. Cancer of the head and neck has an incidence of 23/100,000/ year for males and 8/100,000/year for females. Incidence has increased between 1990 and 2000 in men by 17.4% and in women by 20.2% (Scottish Cancer Registry 2004) Survival overall and in particular for certain age ranges appears to be static or possibly worsening. Simultaneously, there is a rising incidence of this disease in the UK and more markedly in Scotland (Scottish Cancer Registry 2004), especially among younger age groups and in women which is occurring against a general reduction in incidence for cancer generally. These survival trends are seen both for head and neck cancers overall (Table 1) and for oral cavity cancer (Table 2).

There is therefore a clear need for new insights into SCCHN in order to establish new therapeutic strategies. Scotland is a worldwide hotspot for SCCHN with an incidence which has risen to 25/100,000 new case per head of population per year. (Scottish Cancer Registry 2004) Research into this disease carried out here is therefore both highly relevant to the health needs of the population and is facilitated by the population disease burden. Also, any therapeutic advances offered by success in this field will have relatively greater impact in Scotland.

Table 1 Relative Survival at five years for head and neck cancer in Scotland for patients aged 15 – 99 years

	1977-1981	1992-1996
Males	57.6%	47.7%
Females	52.6%	52.2%
Both sexes	56.7%	48.5%

Table 2. Relative Survival at five years for oral cavity cancer in Scotland for patients aged 15 – 99 years.

	1977-81	1992-1996
Male	35.1%	42.0%
Female	41.6%	50.5%
Both sexes	36.1%	43.4%

1.3 Current therapy for SCCHN

The management of SCCHN involves surgery, radiotherapy, chemotherapy and combinations of these modalities. Factors influencing the optimum treatment include features of the primary tumour such as size, site and tumour thickness and patient factors such as age and general medical condition. In the management of early disease, surgery and radiotherapy for the primary tumour show comparable effectiveness in terms of treatment end point (5 year survival rates). Other factors, such as site, stage and neck node status then become significant in decision making. Further, the complication profile of each of these modalities differs and so broader quality of life considerations determine the optimum management plan.

In patients presenting with more advanced disease combined modality treatment is considered. These include larger primary lesions, i.e. T3 and T4 and those with clinically apparent spread of disease to the cervical nodes. These account for 66% of the SCCHN patients presenting in the West of Scotland (Currie, Hislop and Carton in press).

In general for early stage disease, surgical resection or radiotherapy for the primary cancer with appropriate surgical management of the neck is accompanied by adjuvant radiotherapy where indicated. Further, where the resection margin of the primary tumour has been necessarily close, or where there are adverse histological features either at the primary site (e.g. perivascular or perineural invasion) or in the neck (e.g. nodal involvement at multiple levels or extracapsular spread) adjuvant radiotherapy is employed to treat any residual microscopic disease deposits. Clearly, recurrence of a clone of cancer cells is possible with the persistence of a single viable tumour cell.

Standard radiotherapy regimen comprises a total of 60Gy of radiation delivered in fractions of 2Gy five days per week for six weeks. Ionising radiation has a significant side effect profile, including painful mucositis and skin changes, dysphagia, xerostomia, lassitude and

taste problems. Patients often require hospital admission to correct dehydration and nutritional status and there is a small but significant mortality with this treatment.

In advanced disease, or for patients deemed unfit for surgery on medical grounds, combination primary chemoradiation may be utilised (cisplatin and fluorouracil with teletherapy radiation). Significant advances have been made using this treatment modality in comparison to radiotherapy alone in patients with disease considered unresectable. (Forastiere, Goepfert et al. 2003) Some new evidence emerging from trials involving a third agent are yielding promising initial data. (EORTC 2004) The role of chemotherapy generally in the management of this cancer at this time remains investigational.

The overall unchanging survival in the face of enhancements in modern surgical management for SCCHN delineates the need for new therapeutic strategies for this disease. These are best identified in principle by consideration of the fundamental nature of cancer tissue. This can only be achieved by recognition of the genetic basis of malignancy. Cancer is a disease of the genome, involving changes in genes coding for cell growth, differentiation and apoptosis. Understanding the basis of these changes and their effect on cell biology is the route by which better, targeted therapeutic options will be found and survival rates enhanced.

1.4 Cancer as a disease of the genome

Steadily advancing understanding of cancer over the last 30 years has revealed this to be a genetic disease. (Hanahan and Weinberg 2000) Cancer is a multistep process involving upregulation of oncogenes with dominant gain of function and mutations of tumour suppressor genes with recessive loss of function. While our understanding of the complexity of control mechanisms for cell growth and differentiation progressively increases, the salient properties of the cancer cell have been proposed to be the same six essential features, regardless of tissue of origin (Figure 1-1) (Hanahan and Weinberg 2000)

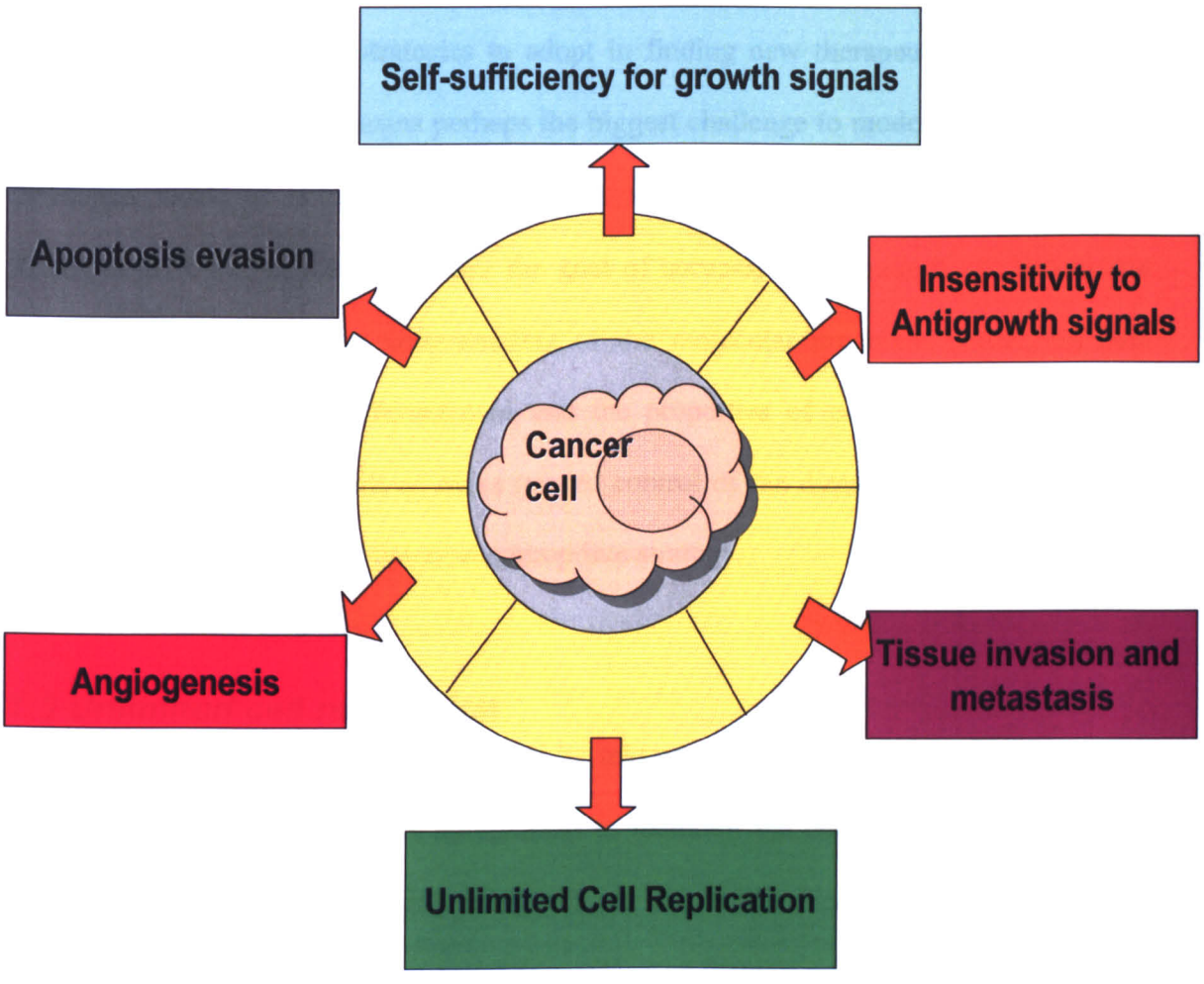


Figure 1-1. Acquired features of Cancer cells (modified from Hanahan and Weinberg 2000.)

Precise control mechanisms underlying these features vary between different cell types so that while the detail may differ the principles are the same. Organising the characteristics of malignant cells into these six main categories not only provides clarity of understanding of the processes involved in oncogenesis but also provides clear lines along which to attack the cancer cell therapeutically. These are the features which distinguish cancer cells from normal tissue and therefore provide, at least in principle, long sought for specificity in designing cancer treatments. Further, attacking more than one of these salient cancer characteristics simultaneously may produce a synergistic therapeutic benefit.

The concept of the holistic management of the cancer patient must remain central to considerations of the best strategies to adopt in finding new therapeutic approaches. In treating a disease which remains perhaps the biggest challenge to modern medicine in the developed world it is essential to maintain vision regarding appropriate outcomes. Attempts to "cure" cancer have been the goal of successive generations of doctors and scientists. However, as our understanding of the molecular processes underlying both normal cell growth and differentiation and the properties of cancer cells increases, it becomes apparent that a shift of focus toward control of the disease process, rather than absolute eradication, may be the most appropriate strategy.

1.5 Unlimited cell replication

This feature of the cancer cell is achieved by telomere maintaining mechanisms. In most cancers, (85-90%) telomerase is upregulated to maintain the chromosome end caps and renders the cell immortal (Kim, Piatyszek et al. 1994). Telomere maintenance by telomerase provides an attractive target in antitumour therapy and this strategic approach forms the basis for this thesis. The details of the telomere and telomerase function are now discussed.

1.6 The Telomere

1.6.1 Telomere composition

Telomeres are composed of tandem repeats of DNA sequence (TTAGGG)n, repeated many hundreds to thousands of times located at the ends of eukaryotic chromosomes. They have a number of associated proteins and have many functions. These include protecting the chromosome from end to end fusion, recombination and recognition as damaged DNA. (McEachern, Krauskopf et al. 2000) They provide a means for complete replication of chromosomes and contribute to the functional organisation of chromosomes within the nucleus. Capping prevents chromosomes from fusing and their ends being misinterpreted as DNA double strand breaks. Telomeres also act as repositories for DNA repair enzymes

(Blasco, Gasser et al. 1999) and can silence genes placed close to them. (Baur, Zou et al. 2001) While most of the telomere is double stranded, each has an overhanging, single stranded length of DNA on the G-rich, 3' strand. This has been implicated as a crucial component of the telomere which is essential for normal function. (Henderson and Blackburn 1989; Griffith, Comeau et al. 1999; Blackburn 2001) This may participate in the organisation of the telomere end into the T-loop, D-loop structure and is the substrate for telomerase, the cellular reverse transcriptase which can add telomeric DNA to the chromosome ends. Recently, this single stranded stretch of telomere DNA was shown to be eroded in senescent cells. Thus it seems that this is the specific molecular alteration, resulting in the exposure of the eroded 3' G-rich end, triggers the senescent state in mammalian cells. (Stewart, Ben-Porath et al. 2003) However it is still unclear whether erosion of the G-rich overhang itself is the cause or consequence of senescence.

1.6.2 Telomere Structure

The telomere structure is not yet fully understood, but appears to protect the 3' single stranded end from degradation and possibly from addition of repeats by telomerase. (Makarov, Hirose et al. 1997; Shore 1997; Wellinger and Sen 1997; Lingner and Cech 1998; Griffith, Comeau et al. 1999; Blackburn 2000; McEachern, Krauskopf et al. 2000; Dubrana, Perrod et al. 2001) Large duplex loops of DNA have been shown to form at the telomere end (Griffith, Comeau et al. 1999; Munoz-Jordan, Cross et al. 2001) These have been termed T-loops and have an associated smaller D-loop. Sizes of these loops vary considerably in different organisms, leading to the conclusion that this structure itself may not form a functional unit (de Lange 2002). Indirect evidence suggests that the T-loop forms by invasion of the single stranded 3' G-rich overhang back into the duplex DNA of the telomere. While the precise structure is not clear, there is a short segment of single stranded DNA which represents the displaced TTAGGG strand (D-loop) at the point of overhang invasion. (Figure 1-2). Further recent evidence suggests that telomere length

homeostasis is achieved via a switch between telomerase-extendable and –nonextendable states. (Teixeira, Arneric et al. 2004)

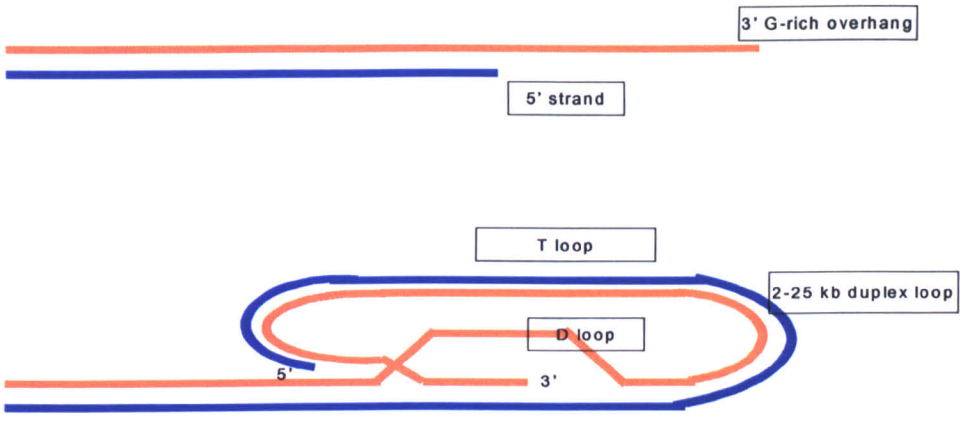


Figure 1-2. Schematic diagram of T-loop D-loop formation at the mammalian telomere modified from Griffith et al 1999

1.6.3 Telomere associated proteins

A number of proteins have been identified which are associated with mammalian telomeres. These have differing roles and many function to regulate telomere length and structure and to protect the telomere. (de Lange 2002) The telomere also binds proteins involved in DNA repair. These include the three components of the DNA dependant protein kinase (DNA-PK) heterotrimer (Ku 70, Ku 80 and DNA-PKcs) (Critchlow and Jackson 1998) and the RMN complex (Mre11, Rad50 and NBS1). Proteins involved in double strand break repair by non-homologous end joining are of particular interest in the context of cancer therapy. The double strand break is the lethal chromosomal lesion produced by ionising radiation and by certain chemotherapeutic agents (see on).

1.7 The Telomerase enzyme.

In the germ line telomere loss is countered by the presence of telomerase (Greider and Blackburn 1985) which perfectly balances the loss of nucleotides during cell division by the direct addition of TTAGGG DNA repeats to the chromosome end (Allsopp, Vaziri et al. 1992). Telomerase is a RNA dependant DNA polymerase which is downregulated at around 20 weeks of intrauterine life in somatic cells. Since its initial discovery in Tetrahymena thermophila in 1985 (Greider and Blackburn 1985) it has been the subject of detailed research which reveals that it is composed of at least two essential components, the RNA template, TERC (Feng, Funk et al. 1995) and the catalytic subunit, TERT. (Nakamura, Morin et al. 1997) One component (hTERC) is expressed in all tissues, (Avilion, Piatyszek et al. 1996) but shows an approximately five-fold increase in expression in cancer cells. (Yi, Tesmer et al. 1999) In contrast the catalytic component (hTERT) is subject to close control and this is thought to be the molecular basis for control of enzyme activity. (Cong and Bacchetti 2000) In addition to the maintenance of telomeres in the germ line by telomerase there is now evidence to indicate that the final length of the telomere is under very sophisticated control. In particular, telomere length in the germ line can sometimes be reset by as yet undefined mechanisms when animals are cloned from senescent somatic cells. (Lanza, Cibelli et al. 2000)

There is some evidence accumulating that telomerase may have roles beyond simple maintenance of telomere length. (Blasco 2002) Specifically, it is suggested that telomerase might promote or increase the rate of cellular proliferation, (Gonzalez-Suarez, Samper et al. 2001) or enhance cell survival. (Mattson, Fu et al. 2001)

1.8 The End Replication Problem.

This term describes the process by which telomeric DNA is lost with every round of cell replication. (Watson 1972) Synthesis of DNA in the 5' lagging strand requires the use of a RNA primer, which is removed leaving a gap which DNA polymerase cannot fill. This leads to attrition of the telomere with between 50-200 nucleotides being lost at each cell division. Further digestion of the strand by a putative 5'-3'- exonuclease is proposed to increase the size of the gap (Makarov, Hirose et al. 1997), so that the double stranded DNA molecules have a 3' single-strand overhang. Repetition of these processes results in progressive attrition of the 5' ends, thus leading to a gradual shortening of the telomeres after many cell divisions. This is shown diagrammatically in Figure 1.3. Telomeres can also be shortened by oxidative damage. (von Zglinicki, Saretzki et al. 1995)

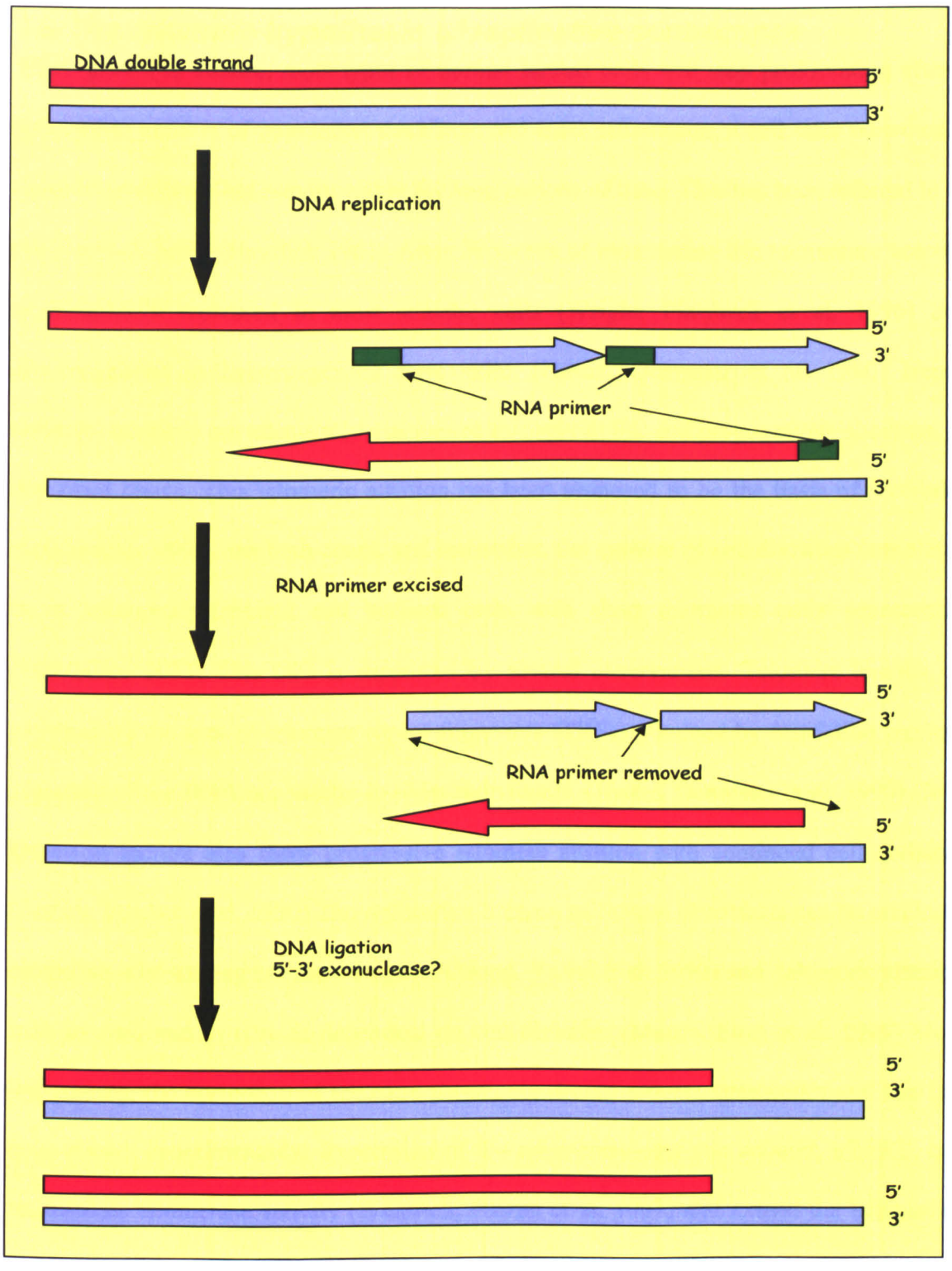


Figure 1-3. The end replication problem and telomeric attrition.

From (McCaul, Gordon et al. 2002). Removal of the RNA primer (green segment) leaves a shortened DNA strand. Further shortening of the 5' end (Red strand) then occurs via putative exonuclease activity, leaving the 3' singlestranded G-rich overhang. Thus the human chromosome shortens at every round of cell replication.

1.9 The telomere hypothesis of replicative senescence

When grown in culture, most types of normal human cells will stop proliferating after a predictable number of population doublings and enter senescence; a cell state where cells cease to proliferate but remain viable for long periods of time. This has been referred to as the Hayflick limit. (Hayflick 1961) After 20 weeks of intrauterine life telomerase activity is completely repressed in most somatic cells (Wright, Piatyszek et al. 1996) and downregulated in haematopoietic stem cells. (Hastie, Dempster et al. 1990) Hence telomere length is not adequately maintained throughout life and progressively shortens, as described above. This telomeric attrition has been proposed to be the basis of a cellular replicometer which can both count, and remember, the number of cell divisions completed by a telomerase-deficient cell because cells with short telomeres enter senescence. (Olovnikov 1973) This idea is supported by several observations. Telomere lengths, as assessed by the size of telomere repeat fragments (TRFs) obtained by restriction enzyme digestion of the DNA are shorter in older individuals. (Hastie, Dempster et al. 1990) Cells grown in culture also show progressive telomere attrition with continued cell division. (Harley, Futcher et al. 1990) The replicative lifespan of human fibroblasts can be predicted on the basis of starting telomere length (Allsopp, Vaziri et al. 1992) and telomeric attrition both *in vitro* and *in vitro* is dependent on cell division. (Munro, Stott et al. 1999) Most importantly, the correction of telomere shortening should prevent senescence and this has been shown experimentally. Expression of the telomerase catalytic subunit, hTERT, can reconstitute telomerase activity (Weinrich, Pruzan et al. 1997) and extend the lifespan of human fibroblasts (Bodnar, Ouellette et al. 1998) without causing karyotypic changes or features of transformation. (Morales, Holt et al. 1999) Mouse models have provided valuable insight into this area. Although laboratory mice (*mus sprutus)* have long and heterogeneous telomeres, (Kipling and Cooke 1990) the cloning of the first essential telomerase component, TERC, (Feng, Funk et al. 1995) has made possible the creation of strains of telomerase-deficient mice (TERC-/-). These show telomeric attrition through successive generations. (Blasco, Lee et al. 1997) Breeding TERC -/- mice through four to

six generations causes compromised proliferation and loss of viability of the germ line and lymphocytes, (Lee, Blasco et al. 1998) sterility, (Lee, Blasco et al. 1998) poor wound healing (Rudolph, Chang et al. 1999) and many features of human ageing *in vitro.* (Rudolph, Chang et al. 1999). Further insights have been gained from disease states where telomerase and hence telomere function is adversely affected. In humans a mutation in the dyskeratosis congenita gene product, dyskerin, leads to an instability of the hTERC transcript and to a reduction in telomerase activity. (Mitchell, Wood et al. 1999) The fibroblasts from these patients cannot be immortalised by the expression of the hTERT gene because there is too little hTERC to generate the required enzyme activity. Interestingly, dyskeratosis congenita is characterised by early onset anaemia and bone marrow failure resembling the problems encountered by the telomerase null mice at later generations. Therefore, collectively, these studies support the concept that telomeric attrition prevents indefinite clonal proliferation *in vitro.*

1.10 Human cellular mortality and the role of telomeres.

Most somatic human cells, including the keratinocytes of squamous epithelium, are telomerase deficient and mortal *in vitro.* In cultured epithelial cells replicative senescence is thought to be controlled first by the accumulation of p16INK4A (mortality mechanism 0 or M0). M0 is not directed by telomere length (Ramirez, Morales et al. 2001) but it has been suggested that M0 is dependent on the rate of oxidative damage, the cell type and the culture environment and that putative telomere-independent mechanisms of replicative aging might reflect inadequate growth conditions. (Ramirez, Morales et al. 2001) However, it should be emphasised that although there is evidence to show that oxidative damage drives the accumulation of p16INK4A, or M0 *in vitro*, the role of M0 as an *in vivo* checkpoint to the development of cancer has not been firmly established. (Forsyth, Evans et al. 2003; Parrinello, Samper et al. 2003) After further multiple divisions and a period of telomere loss these cells then encounter mortality mechanism 1 (M1) (Wright, Pereira-Smith et al. 1989) (see Figure 1-4). M0 and M1 are biologically indistinguishable

(Romanov, Kozakiewicz et al. 2001); both are cell cycle arrest states and referred to as replicative senescence. Thus loss of telomere length mediates the cessation of cell division. The M1 checkpoint is mediated by p53 and may be triggered by exposure of the 3' end following disruption of the t loop. (Blackburn 2001) In fact, t loop disruption by the expression of a dominant negative TRF2 induces a growth arrest with features of senescence. (van Steensel, Smogorzewska et al. 1998) Most recently, evidence of erosion of the 3' single strand DNA overhang has been demonstrated in senescent cells and this specific change has been suggested to be the trigger for growth arrest. (Stewart, Ben-Porath et al. 2003) Indeed using T-oligos to mimic exposure of the 3'- end has been shown to rapidly induce senescence in malignant fibroblasts. (Li, Eller et al. 2004) Following disruption of the cell cycle control pathways (and M0 and M1) by for example, DNA tumour viruses, the telomeres shorten still further, until one or more becomes dysfunctional. At this time, as a consequence of telomeric fusions, many dicentric chromosomes and anaphase bridges form and result in a considerable amount of cell death. (Counter, Avilion et al. 1992; Counter, Hahn et al. 1998) This checkpoint on the road to cellular immortality is known as crisis or mortality mechanism 2 (M2) (Wright, Pereira-Smith et al. 1989) (see Figure 4). From these unstable cultures, rare variant cells emerge (1 in 10^7) which have acquired unlimited replicative potential. These cells have progressed to a state where telomeres are maintained and in 85-90% of cases this is by upregulation of telomerase. (Counter, Avilion et al. 1992) However, less commonly, telomerase negative cells survive crisis and become immortal because they use a recombination-based telomere elongation mechanism known as ALT (Alternative Lengthening of Telomeres). (Bryan, Englezou et al. 1997) While ALT has never to date been demonstrated in SCCHN, its possible relevance to the successful treatment by telomerase inhibitors is discussed below. The role of telomerase deficiency in human cellular mortality is demonstrated by the experiments which show that the ectopic expression of telomerase can bypass both

senescence (Bodnar, Ouellette et al. 1998) and crisis (Counter, Hahn et al. 1998) in these models.

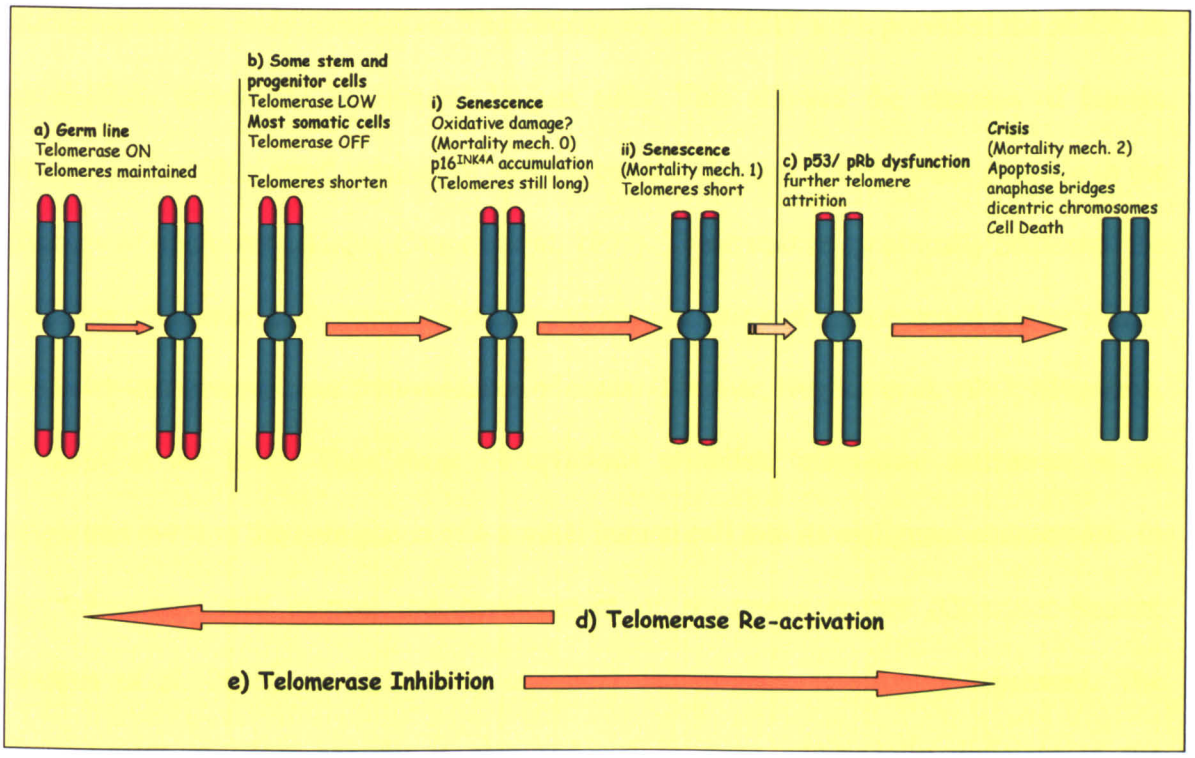


Figure 1-4. The telomere hypothesis of replicative senesence and cellular mortality.

a) There is now considerable evidence that telomerase is active in the germ line and that it maintains the telomeres through successive generations. b) At some point during development telomerase is down-regulated, or extinguished in most somatic human cells from that time telomeric attrition is proposed to begin. Most carcinomas first encounter the telomere length-independent mortality mechanism 0 (i), which is bypassed by the loss of p16INK4A early in SCCHN development. Telomere shortening continues and culminates in replicative senescence or mortality mechanism 1 (ii). c) The elimination of the p53 and pRb pathways by DNA tumour viruses or by somatic mutations in the pathways themselves allows the cells to tolerate short telomeres and divide further. In the continued absence of telomerase this results in further telomeric attrition until the telomeres become dysfunctional and the cells die as a consequence of mitotic failure precipitated by dicentric chromosomes and anaphase bridges. d) The re-expression or up-regulation of telomerase adds TTAGGG repeats to the dysfunctional telomeres, renders them functional again and allows the cells to bypass crisis, or mortality mechanism 2. e) Conversely the inhibition of telomerase drives telomerase-positive cells back into crisis.

1.11 Telomere function and the development of cancer.

While studies had shown that the great majority of malignant cells have upregulated telomerase, until recently this evidence associating telomerase with human telomere maintenance was only correlative. The cloning of the hTERT gene provided the ability to reconstitute telomerase in cultured human cells. This allowed the creation of human tumour cells with a small number of defined genetic changes, which is not possible in the absence of telomerase (Hahn, Counter et al. 1999). These studies specifically addressed the function of telomerase in immortalisation and oncogenesis and demonstrated a clear role in telomere maintenance and the avoidance of crisis. (Lingner, Hughes et al. 1997; Meyerson, Counter et al. 1997) Thus these observations establish telomerase activation as an important event in the conversion of a normal human cell into its malignant counterpart. In the telomerase null mouse, the development of squamous cancer (Gonzalez-Suarez, Samper et al. 2000), as elicited by chemical carcinogens, is strongly repressed. The development of other cancers is also reduced in mice additionally deficient in the Adenomatous Polyposis Coli (APC) (Rudolph, Millard et al. 2001) and INK4A-/- (Greenberg, Chin et al. 1999) tumour suppressor genes. However, when short telomeres are combined with p53 deficiency, an increased tumour yield is observed (Chin, Artandi et al. 1999) and tumours with cytogenetic features (unbalanced translocations, Robertsonian translocations, regional gains and losses) reminiscent of their human counterparts are observed (Artandi, Chang et al. 2000). The generation of genetic instability by dysfunctional telomeres and the acceleration of cancer development is speculated to occur during a short period, in which many human cancer cells will die as part of crisis (Artandi and DePinho 2000). Once telomeres become functional again, due to the up-regulation of telomerase, the genetic stability of the tumours is predicted to be restored. It is certainly plausible that in human tumours the genetic instability generated during crisis contributes to the up-regulation of telomerase. The telomerase gene is in fact located on the short arm of chromosome 5 and within 2Mb of the chromosome end (Chr5 p15.33). The combination of short dysfunctional telomeres and p53 mutation is likely to exist in the latter stages of

development of many human cancers. (Hastie, Dempster et al. 1990; Brachman, Graves et al. 1992) One caveat to the telomerase inhibition strategy is the possibility that this might accelerate the development of subsets of tumours that can use ALT mechanisms to maintain telomeres, or even uncover ALT function in cells where both mechanisms coexist. (Gan, Mo et al. 2002) Interestingly, however, in some cancers which are typically telomerase negative and telomeres are therefore maintained by the ALT mechanism, telomerase expression is associated with increased tumour aggressiveness. (Sanders, Drissi et al. 2004) these issues are discussed in more detail in section 1.13.

1.12 SCCHN immortality and the role of telomerase.

The immortal phenotype can arise early in SCCHN progression (McGregor, Wagner et al. 1997; McGregor, Muntoni et al. 2002)but generally is more prevalent in the more advanced stages of the disease. (Edington, Loughran et al. 1995) Immortal SCCHN cells almost always possess dysfunctional p16INK4A, p53 and retinoic acid receptor β genes (Loughran, Clark et al. 1997; McGregor, Wagner et al. 1997) and these markers are associated with the bypass of M1. The bypass of M2 is most likely achieved by the up-regulation of telomerase which is ubiquitous in SCCHN lines and there are no reports to date of ALT pathway activation. (Loughran, Clark et al. 1997) These characteristics are found in a large subset of SCCHN *in vitro* (Brachman, Graves et al. 1992; Kim, Piatyszek et al. 1994; Reed, Califano et al. 1996) and the size of this subset matches very closely the fraction of SCCHN tumours that are immortal *in vitro*. (Edington, Loughran et al. 1995)

Normal basal squamous keratinocytes do possess detectable *in vitro* telomerase activity (Harle-Bachor and Boukamp 1996) but there is no evidence that this telomerase is functional. In fact, the telomeres of normal squamous epithelium from old individuals (Lindsey, McGill et al. 1991), immortal SCCHN cells (Gordon, Ireland et al. 2003) and squamous cell carcinomas *in vitro* (Parris, Jezzard et al. 1999) are considerably shorter than normal. Also immortal SCCHN lines have cytogenetic changes, such as Robertsonian

translocations (K. G. Edington – unpublished data) that result from the telomeric fusion of two chromosomes (usually acrocentrics). SCCHN lines also have numerous chromosomal gains and losses, which together with Robertsonian translocations are consistent with the SCCHN cells having passed through crisis. (Artandi, Chang et al. 2000; Artandi and DePinho 2000) Finally, recently obtained direct evidence suggests that post M1, pre M2 SCCHN cells exist and are present *in vitro*. (Gordon, Ireland et al. 2003) SCCHN cells with mutant p53, deleted INK4A, short telomeres and low telomerase can be detected *in vitro* and these cells display features of crisis, including dicentric chromosomes and anaphase bridges. The ectopic expression of telomerase in these cells reduces dicentrics and anaphase bridges and improves growth. Furthermore, some SCCHN tumours have high anaphase bridge indices *in vitro* and comparisons of telomere function between primary cancers and neck metastases show tumour progression to evidence of reduced levels of telomere dysfunction. Taken together, this data is consistent with the idea that SCCHN cells re-activate telomerase following an extensive period of telomere shortening during multiple cell divisions.

Thus the telomeres in SCCHN generally are very much shorter than the telomeres in normal somatic cells even in older patients. This should ensure that following telomerase inhibition, telomere dysfunction should ensue and produce cell death selectively in the cancer tissue before any normal tissue toxicity is observed (see below).

1.13 Telomerase as a therapeutic target

A variety of different strategies targeting telomerase have been proposed and investigated. Telomerase is in fact unusual in that a substantial body of basic scientific research has been carried out prior to the development of effective drug compounds rather than the reverse situation. This has allowed the anticipation of many potential problems which is the opposite circumstance to that for most drug development (Shay and Wright 2002). Importantly, the ability of telomerase inhibition to kill tumour cells does not require the p53 (Hahn, Stewart et al. 1999) or the INK4A (Zhang, Mar et al. 1999) genes to be intact and this is significant, given the frequency of the inactivation of these genes in human tumours.

There are some potential difficulties with the telomerase/ telomere function target for cancer therapy.

First, many strategies inhibiting the enzyme will require a lag phase, during which further cell division and hence telomere shortening will occur prior to cell death. This lag phase will be proportional to the starting telomere length. (Feng, Funk et al. 1995; Hahn, Stewart et al. 1999; Herbert, Pitts et al. 1999; Zhang, Mar et al. 1999) Human tumours have been shown to have very short telomeres but some further tumour growth (and progression) would occur with this treatment.

Secondly, some normal tissues express telomerase, including germ cells, haematological cells and basal cells of the epidermis and intestinal crypts. These normal tissues however only transiently express the enzyme and so any effect here would be less marked than in cancer cells. Clearly the planned duration of antitelomerase therapy would be significant in anticipating this type of toxicity.

The inhibition of telomere maintenance by targeting telomerase will certainly produce selective pressure for the emergence of other mechanisms of telomere maintenance. Other means by which telomeres can be maintained are known to exist in cell lines which are

telomerase negative and have very heterogeneous telomere lengths. Termed the ALT pathway (Alternative Lengthening of Telomeres), the precise mechanism remains unclear but includes one thought to depend on homologous recombination, in both yeast and mammals. (Lundblad and Blackburn 1993; Bryan, Englezou et al. 1995; Bryan, Englezou et al. 1997; Bryan and Reddel 1997; Reddel, Bryan et al. 2001; Henson, Neumann et al. 2002; Lundblad 2002) Recent evidence suggests that sister chromatid exchanges of the telomeric DNA occur at high rates in some ALT lines, which, if unequal may facilitate unlimited cell replication. (Bailey, Brenneman et al. 2004) This mechanism is however rare in human cancer and immortal tumour cell lines generally and represents only 6-7% of the samples analysed to date. (Reddel, Bryan et al. 2001) Furthermore there is no evidence that ALT is used to maintain untreated SCCHN telomeres. (Loughran, Clark et al. 1997) Another possible area of concern connected to the ALT mechanism is dysfunctional mismatch repair. In yeast this has been shown to facilitate ALT in telomerase deficient strains and many human tumours resistant to various chemotherapy regimens lack efficient mismatch repair pathways. (Rizki and Lundblad 2001) This issue has not however been extensively investigated in SCCHN and until recently the only published study did not find any significant selection for cells deficient in mismatch repair pathways after chemotherapy. (Blons, Cabelguenne et al. 1999) One recent report however has now shown that telomerase inhibition in a mismatch repair deficient colorectal carcinoma line can produce ALT-like telomere elongation. (Bechter, Zou et al. 2004)

The concern has also been raised that the crisis phenotype produced by telomere shortening might lead to the emergence of more aggressive tumours. Although some evidence supports this concern, (Chin, Artandi et al. 1999) (Gonzalez-Suarez, Samper et al. 2001) the majority of the available evidence suggests the opposite and so is reassuring in this regard. (Herbert, Pitts et al. 1999; Zhang, Mar et al. 1999; Kim, Rivera et al. 2001) (Greenberg, Chin et al. 1999; Gonzalez-Suarez, Samper et al. 2000; Boklan, Nanjangud et al. 2002) (de Lange and Jacks 1999; Hahn, Stewart et al. 1999; Franco, Segura et al. 2002;

Goytisolo and Blasco 2002) This would be averted by the use of multiple synergistic therapeutic interventions, such as the inclusion of an effective antiangiogenesis strategy. It is clear however, that the emergence of any new cancer in the face of potent telomerase inhibition would require the institution, in a short time frame, of an effective but different telomere maintenance mechanism. This has not yet been seen in SCCHN.

Four broad areas show the most promise for clinical application and these are briefly considered below

1.13.1 Telomerase specific oncolytic virus

The high frequency of telomerase activation in cancer cells specifically allows the design of therapy based on the hTERT proximal promoter. Regulation of hTERT at the transcription level means that only telomerase positive cells activate this promoter region. Genes coding for pro-apoptotic pathways have been transferred using direct intratumour injections of plasmids or adenoviral vectors. These contain the hTERT promoter upstream from genes such as caspase 6 and 8 (Komata, Kondo et al. 2001), Bax (Gu, Kagawa et al. 2000) and the FADD gene (Koga, Hirohata et al. 2001). Replication deficient adenovirus is however limited as a cancer therapy due to limited transduction of tumour tissue. A recent development has been the production of tumour-specific replication competent adenoviral gene therapy (hTERTp-TRAD). (Shay and Wright 2002) This adenovirus infects both normal and malignant cells, but only replicates in and subsequently lyses cells in which telomerase is expressed. Intact virions of hTERTp-TRAD would then infect adjacent tumour cells amplifying the effect. With this approach there is no lag phase and so cytotoxicity is immediate. This may mean however that cells which transiently express telomerase (spermatocytes, intestinal crypt cells and basal and suprabasal epidermis and mucosa) would also be damaged. (Holt and Shay 1999; White, Wright et al. 2001; Forsyth, Wright et al. 2002)

The transcriptional regulatory sequences of hTERC and hTERT have also been used to activate expression of the bacterial nitroreductase enzyme. Used in combination with a

prodrug (CB1954) cells have been shown to be sensitized and efficiently killed. (Boyd, Mairs et al. 2001; Plumb, Bilsland et al. 2001; Bilsland, Anderson et al. 2003)

1.13.2 hTERT immunotherapy.

T cells targeted to tumour antigens have been shown to produce tumour regression. (Vonderheide 2002) The catalytic component of human telomerase (hTERT) is processed intracellularly and peptide fragments are presented as epitopes by MHC. This produces protective immune responses against tumours. (Minev, Hipp et al. 2000; Nair, Heiser et al. 2000; Vonderheide 2002) Again in this approach the widespread and vast overexpression of telomerase in many cancers relative to normal tissue shows the potential range and effectiveness of the telomerase target. Here also the lag phase is avoided, but the possibility of an adverse effect on normal tissue transiently expressing telomerase could occur. Specific CTL cell kill has been elicited in prostate, lung, breast, colon and melanoma cells, but not in $CD34^+$ haemopoietic stem cells. Thus the far higher and more persistent expression of telomerase in cancer may lend an adequate degree of specificity to this strategy. (Vonderheide 2002)

1.13.3 Anti hTERC strategies.

The RNA component of the telomerase holoenzyme complex has been targeted with several classes of oligonucleotides. These include phosphdiester DNA, phosphromidates, peptide nucleic acids, locked nucleic acids, 2'O-methyl-RNA, 2'-O-methoxyethyl RNA. (Norton, Piatyszek et al. 1996; Pitts and Corey 1998; Hamilton, Simmons et al. 1999; Corey 2000; Elayadi, Demieville et al. 2001; Gryaznov, Pongracz et al. 2001; Corey 2002; Herbert, Pongracz et al. 2002; Asai, Oshima et al. 2003) This approach leads to progressive telomere shortening in intact cells, thus incurring a lag phase in therapy. As with many gene therapy approaches, the above strategies are dependent on the development of suitable delivery systems which do not currently exist. Another anti-hTERC strategy described recently uses a lentiviral delivery system for efficient overexpression of mutant

template RNA. This approach rapidly inhibited cell growth and induced apoptosis in telomerase positive precancer and cancer cells. (Li, Rosenberg et al. 2004)

1.13.4 Antitelomerase compounds

Experiments using the expression of dominant negative telomerase mutants have clearly shown the ability to kill cancer cells with this approach. (Hahn, Stewart et al. 1999; Zhang, Mar et al. 1999) Again the problems of delivery to the tissues limits the usefulness of such a strategy clinically.

Drugs that have been shown to inhibit telomerase include reverse transcriptase inhibitors (including AZT), nucleoside analogues, isothiazolone derivatives, a rhodocyanine and a catechin component of green tea (see Kelland for a review (Kelland 2001)). A further class of agents are those that target G quadruplex (or tetraplex) structures rather than duplex DNA. (Kelland 2001) Although there is no evidence to support the existence of quadruplex structures in mammalian telomeres, quadruplex structures are formed by the G strand overhang at the telomere *in vitro* and the aim of the G quartet-interacting drugs is to block the interaction between telomerase and its substrate. (Kelland 2001)

The problem with most of the above drugs is that they affect the growth of telomerase-negative cells (Rha, Izbicka et al. 2000) (Naasani, Seimiya et al. 1999) suggesting that they may influence biological processes other than telomerase activity. However, more recently, a new non-nucleosidic drug, designated BIBR1532, was shown to inhibit telomerase, elicit telomere reduction and trigger cell cycle arrest in a highly selective manner, in a number of immortal human cancer cell lines (Damm, Hemmann et al. 2001). At the same concentration, the drug did not affect telomerase-negative normal cells or immortal cells using the ALT mechanism of telomere maintenance. BIBR1532 can also be administered orally and can inhibit the growth of human tumour xenografts in nude mice and as such represents the most promising anti-telomerase agent discovered to date. The mechanism of action of BIBR1532 has been clarified more recently (Pascolo, Wenz et al. 2002) showing it to act non-competitively and similarly to non-nucleosidic inhibitors of

HIV1 reverse transcriptase. Other reports about this agent have been less encouraging, suggesting that IC(50) values may be higher than previously reported and that further refinement is necessary. (Barma, Elayadi et al. 2003)

Although cryptic damage, in the form of telomere reduction, may be sustained by normal telomerase positive cells, the available evidence suggests that on removal of telomerase inhibition, the telomeres return to their original length. (Herbert, Pitts et al. 1999) This result encourages the belief that normal cells may be able to reset their telomere lengths once anti-telomerase therapy has been curtailed. Hence, any effect of a telomerase inhibitor agent of shortening telomeres in either haematological or germ cells will only occur during the period of drug administration and it is likely that telomeres will be restored when therapy stops. Clearly, if long term therapy were to be considered then effects on normal tissue may become relevant but if a shorter duration, perhaps in combination with other treatment strategies were to be employed this would be less so. This opinion is also supported by the ability of senescent bovine cells to produce cloned animals with normal telomere lengths. (Lanza, Cibelli et al. 2000)

1.14 Telomere dysfunction and radiosensitisation.

Ionising radiation is an important component of the overall management strategy for treatment of SCCHN. The effectiveness of radiotherapy lies in the greater sensitivity of cancer tissue over normal tissues, the exact basis of which remains unclear.

1.14.1 The biological effects of ionising radiation

Therapeutic radiation can be either electromagnetic: x-rays or gamma rays, or particulate: electrons, neutrons and protons. These vary in terms of the rate of deposition of energy per unit length of tissue. This is termed linear energy transfer (LET) and therefore the same dose of radiation from sources of differing LET have different relative biological effectiveness (RBE). The deposition of energy in the tissues causes cell death principally by damaging DNA, which is the critical intracellular target. This occurs by two main mechanisms; direct and indirect action.

Direct action of radiation describes the process where the atoms of the molecular target (DNA) are themselves ionised, producing a biologic change. This is more marked in high LET radiation.

Indirect action is where radiation interacts with other cellular constituents (principally water, which comprises 80% of the cell contents) causing ionisation events which produce free radicals. These free radicals are uncombined atoms or molecules carrying an unpaired electron in the outer shell and are highly chemically reactive. Interaction with nuclear DNA then produces molecular damage to sugar and base molecules, crosslinks between DNA strands and also single and double strand breaks. This mechanism predominates in low LET radiation (such as x-rays or gamma rays) and therefore predominates in therapeutic irradiation for SCCHN.

One gray (Gy) of ionising radiation is estimated to produce approximately 150,000 ionisation events in the nucleus. Only about 1,000 of these are DNA strand breaks. The double strand break (DSB) is the lethal chromosomal damage produced by ionising radiation. (Vamvakas, Vock et al. 1997) and these are created at a rate of approximately one DSB per chromosome per Gy of radiation. Thus a standard treatment fraction of 2Gy will produce on average two DSBs per chromosome per exposed cell. Ultimately failure to repair DSBs results in loss of cellular reproduction and cell death.

1.14.2 Cellular mechanisms for DSB repair.

The two major mechanisms involved in DSB repair in mammalian cells are nonhomologous end joining (NHEJ) (Jeggo 1998) and homologous recombination (HR). (Johnson, Liu et al. 1999) HR is an error free process and is relatively rare in mammalian cells. In contrast, NHEJ is relatively error prone and may well be responsible for many of the mutations seen in cells exposed to radiation.

Evidence from yeast studies shows that DSB repair proteins are stored at the telomeres and are mobilised from there to the site of DNA damage. (Martin, Laroche et al. 1999; Mills, Sinclair et al. 1999) In mammalian cells, Ku proteins are bound at the telomere (Bianchi and de Lange 1999; Hsu, Gilley et al. 1999) and prevent end to end fusion and guide localisation of the chromosome within the nucleus. (Samper, Goytisolo et al. 2000) These proteins also form the DNA end binding components of the DNA dependant protein kinase (DNA-PK) heterotrimer which is essential for NHEJ. (Smith and Eck 1999) The DNA repair RMN complex (Rad50/Mre11/NBS1) is also bound at the telomere (due to an interaction between NBS1 and TRF1 (Wu, Lee et al. 2000) and TRF2. (Zhu, Wang et al. 1999))

1.14.3 The mammalian telomere in radiosensitivity

As well as the localisation of DNA repair proteins at the telomere, further information on the role of the telomere in chemo- and radio-sensitivity has recently emerged. (Goytisolo, Samper et al. 2000; Lee, Rudolph et al. 2001; Ludwig, Saretzki et al. 2001) (Wong, Chang et al. 2000) When telomerase is inactivated in mice by the targeted disruption of the mouse TERC gene, the telomeres progressively shorten with each generation until a radiosensitivity syndrome is imparted, producing accelerated mortality on exposure to ionising radiation. (Wong, Chang et al. 2000) In these experiments it was possible to demonstrate that the radiosensitivity was related to telomere dysfunction rather than the mere absence of telomerase (Figure 4). At the cellular level, crypt stem cells in the gastrointestinal tract and primary thymocytes in TERC-/- mice were shown to have increased rates of apoptosis. Furthermore, mouse embryonic fibroblasts had a diminished clonogenic survival. This cellular level radio-sensitivity was shown to correlate with the persistence of complex chromosomal aberrations and retarded DNA repair kinetics, showing an intimate relationship between intact, functioning telomeres and the cellular and whole organism response to ionising radiation. (Wong, Chang et al. 2000)

The degree of radio-sensitivity of cell lines has also been correlated with telomere length. When mouse lymphoma cells sensitive to ionising radiation were compared to radio-resistant parental cells, (McIlrath, Bouffler et al. 2001) a seven-fold difference in TRF length was shown between the cell types. This has been extended to human cells where a significant inverse correlation between telomere length and chromosomal radiosensitivity was shown in lymphocytes from 24 breast cancer patients and five normal individuals. (McIlrath, Bouffler et al. 2001) Goytisolo et al have also shown a correlation between telomere length and radiation sensitivity. (Goytisolo, Samper et al. 2000) Here, fifth generation telomerase deficient mice, which had telomeres 40% shorter than wild type control animals, were hypersensitive to ionising radiation. The mice died of acute radiation toxicity in the gastrointestinal tract, lymphoid organs and kidneys and showed greater chromosomal damage and higher numbers of apoptotic cells than the similarly irradiated control animals. This concept is summarised in figure 1-5.

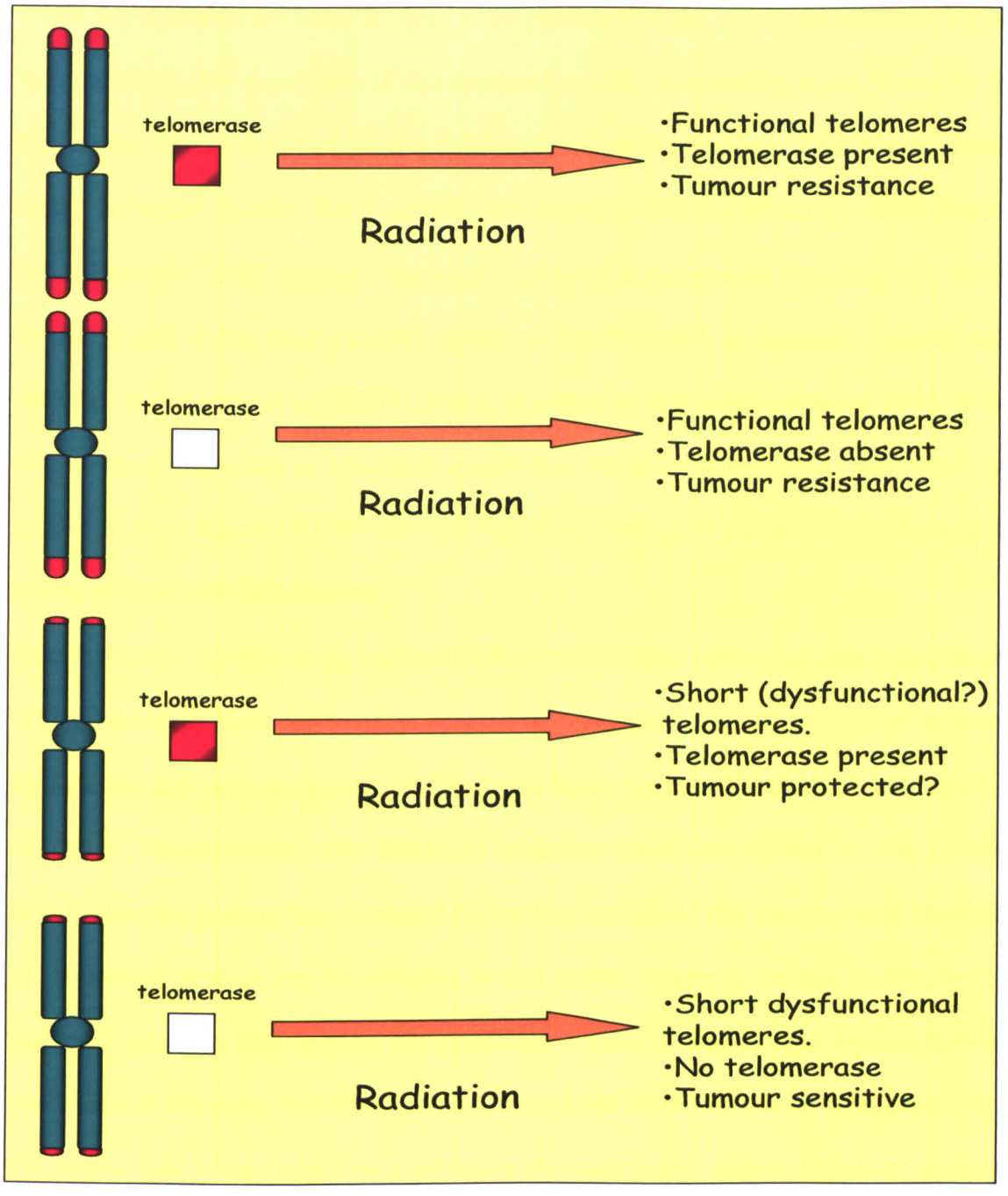


Figure 1-5. Telomere dysfunction radiosensitises cells to ionising radiation.

The figure summarises how telomere length regulates the radio-sensitivity of mouse cells engineered to lack telomerase and possess either short or long telomeres. The presence of telomerase is indicated by the filled boxes and the size of the telomeres by the red portions of the chromosomes. The results so far indicate that cells remain radio-resistant even in the absence of telomerase, provided that the telomeres are long.
The combination of an absence of telomerase and short telomeres, results in telomere dysfunction and increased radio-sensitivity. However, it is not yet clear whether the presence of telomerase alone is enough to protect cells from radiation, even when the telomeres are short.

While the available evidence is clear in suggesting that the shortened telomere imparts radiosensitivity, the exact basis of this increased cellular vulnerability is not. It may be that this shorter telomere cannot form a functional cap or that perhaps DSB repair proteins cannot be bound. Further, the 3'-G rich overhanging strand is a stretch of single stranded DNA. Exposure of this structure has been implicated in senescence signalling (Li, Eller et al. 2003) and it has been recently shown to be shortened in senescent normal cells. (Stewart, Ben-Porath et al. 2003) Clearly, exposure of this single stranded DNA, which will be more vulnerable to ionisation events than double stranded DNA, may result in a greater relative degree of DNA damage signalling leading to increased cell death in the setting of the shortened telomere.

Experiments in the telomerase knockout mouse yield further interesting data in this regard. Where telomeres have shortened in successive generations to reach a point of telomeric dysfunction and radiosensitivity, double strand break repair is impaired. (Latre, Tusell et al. 2003) Chromosomes with shortened telomeres have been shown to fuse to DNA double stranded broken ends produced by ionising radiation. The chromosomal instability of the mouse genome has been shown in this model system to be due to this form of abnormal chromosomal fusion. The implication then is that similar fusions between dysfunctional telomeres and DSBs may be responsible for an increase in radiosensitivity in the cancer cell which might be exploitable therapeutically. (Latre, Tusell et al. 2003) Figure 1-6 summarises this hypothesis.

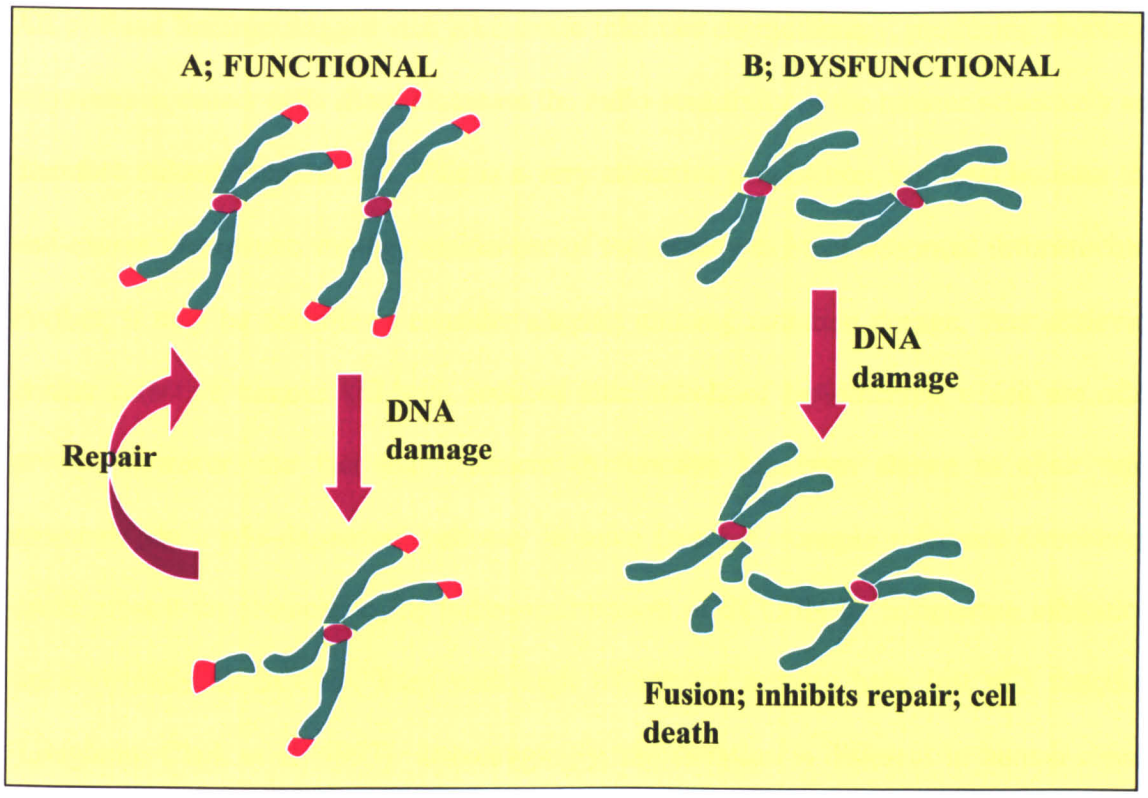


Figure 1-6. Telomere dysfunction and fusions with DSBs.

This figure illustrates findings from experimental work in mice using the telomerase knockout model. In A. capped, functional telomeres protect the chromosome ends from inappropriate fusion when DSBs repair by non-homologous end joining. In B; the uncapped dysfunctional telomere cannot be distinguished from the DSB, resulting in chromosomal fusion, inhibition of accurate DNA repair and loss of cell viability.

All of these findings suggest that telomerase inhibitor chemotherapy, producing shortened telomeres in cancer cells should increase the radio-sensitivity of the tumour selectively and therefore enhance tumour kill. This is a very attractive proposition, not least because this anti-cancer therapeutic strategy makes use of the already existent treatment infrastructure. Further, it may be feasible to consider altering ionising radiation dosage, thus achieving greater selective tumour kill with reduced side effects of radiotherapy which are often severe. However, the fact that telomere dysfunction has been shown to elicit rapid apoptosis via a p53-dependent pathway in mice (see for example reference Greenberg) could present an obstacle to the radio-sensitisation of SCCHN by telomerase inhibition, since virtually all SCCHN lines with high telomerase activity have lost p53 function. (Loughran, Clark et al. 1997) Encouragingly, the situation is different in human cancer cell lines, where in many cases p53 is unnecessary for apoptosis when telomerase is inhibited (Parris, Jezzard et al. 1999). Furthermore, the p53 status (mutated or wild type) of SCCHN cultures does not influence their radiosensitivity. (Brachman, Beckett et al. 1993) However, it is clear that the role of p53 in telomere dysfunction-related phenotypes needs to be examined in SCCHN. Further, the suggested gain of function phenotype of missense mutated p53 requires investigation. (Gasco and Crook 2003)

1.15 Telomere dysfunction and chemotherapy.

Recent evidence from work in oncogene-transformed telomerase-deficient mouse cells has shown that telomere dysfunction rather than the lack of the telomerase enzyme itself is the principal determinant of sensitivity of tumours to certain chemotherapeutic agents. (Lee, Rudolph et al. 2001) The telomerase-deficient transformed cells with short, dysfunctional telomeres were sensitised to killing by drugs inducing the same kind of DNA damage as ionising radiation. Double strand breaks are produced by the anthracyclines doxorubicin and daunorubicin and by actinomycin D but not by drugs working through other mechanisms (cisplatin, etoposide or fluorouracil). Sensitisation correlated with increased chromosomal fragmentation and fusion. Moreover, all the effects were reversed by the re-

introduction of telomerase. This evidence of specific sensitisation of tumour cells highlights one of the principal attractive features of the telomerase/ telomere target. However, the effects were somewhat muted in mouse cells without wild type p53. The introduction of a hammerhead ribozyme into breast tumour cells and an immortal breast cell line produced cleavage of hTERT mRNA and attenuation of telomerase activity, (Ludwig, Saretzki et al. 2001) resulting in sensitivity to doxorubicin. Further, the introduction of telomerase into human fibroblasts reduced sensitivity to the same drug. (Ludwig, Saretzki et al. 2001) As in the first study, sensitivities to other cytotoxic drugs, such as cisplatin were unaffected.

A further significant development is work analysing the effect of simultaneously attacking the telomere and inhibiting telomerase in telomerase positive human cancer cells. (Mo, Gan et al. 2003) Here a synergistic effect was demonstrated using telomerase inhibition together with paclitaxel to shorten telomeres which was specific to telomerase positive cells and not seen in cells using ALT.

These studies suggest that in certain situations the combined use of chemotherapy and telomerase inhibition may be effective against SCCHN. The role of p53 in telomere dysfunction-mediated chemosensitisation in human cancer needs to be investigated (see above).

1.16 The role of p53

1.16.1 p53 in cancer and in SCCHN

First recognised in the 1970s, p53 is now recognised to be a crucial tumour suppressor protein in the mammalian cell and an enormous body of research into this area continues. This function is performed by its role in mediating cellular stress (e.g. hypoxia, DNA damage, oncogene activation) and activating a series of downstream targets which ultimately result in either cell cycle arrest in G1 or in programmed cell death. With a half life *in vitro* of 30 minutes, p53 is modified by external stresses which result in stabilisation of the protein, thus allowing nuclear DNA binding by which its effects are produced.

It is the most commonly mutated gene in many common cancers and is estimated to be mutated in SCCHN in 85% of tumours. Notably, in approximately 90% of cancers, the mutation is missense. (Gasco and Crook 2003)This proportion is very unusually high and has led to speculation regarding selective pressure for this kind of mutation in human cancer. Thus, the suggestion is that there may be a selective advantage, over and above the loss of wild type p53 function, conferred by missense mutation. (Hussain, Hollstein et al. 2000) In fact, some human cancer mutated p53 proteins have been shown to have properties not seen in wild type p53, including increasing resistance to drugs, increased tumorigenicity and the ability to transform cells in cooperation with oncogenes. (Sigal and Rotter 2000) The molecular basis of these effects is unclear but may involve binding of the missense mutant protein with either wild type p53 or other p53 family members such as p63 and p73 thus inhibiting their tumour suppressive effects. These proteins have been shown to bind to p53 DNA-binding sites *in vitro* and can bind to and activate p53 downstream target genes when overexpressed *in vitro*. (Kaelin 1999) In contrast to p53 these proteins are rarely mutated in human cancer although a precise role has yet to be clarified.

In SCCHN the reported p53 mutation rate has varied in several studies (Gasco and Crook 2003) Importantly, some studies may have underestimated the frequency of mutation by analysing only exons 5 to 8 rather than all of the coding DNA (exons 2 to 11). Where all coding sequences have been analysed mutation rates of between 79% to 91% have been found. (Kropveld, Rozemuller et al. 1999; Balz, Scheckenbach et al. 2003)

1.16.2 p53 Codon 72 polymorphism in cancer

In exon four at codon 72 there is a common polymorphism in the p53 protein. This results in the expression of either arginine (R72) or proline (P72) at this codon. This feature shows an intriguing gradient in human populations, with the arginine allele becoming increasingly prevalent with distance from the equator and there has been speculation as to the role of

this allele in coordinating the cellular response to UV induced damage. (Beckman, Birgander et al. 1994)

The arginine form has been shown to be more prone to HPV E6 mediated ubiquitination degradation than the proline form. This leads to the suggestion that individuals with this germline form of p53 may be more susceptible to DNA virus related cancers. (Storey, Thomas et al. 1998) However, studies investigating susceptibility to SCCHN with these polymorphisms have failed to show any increased risk of malignancy. (Hamel, Black et al. 2000; Summersgill, Smith et al. 2000; Shen, Zheng et al. 2002)

In established cancers in patients who are heterozygotes for Arg/Pro at codon 72 the 72P allele is invariably lost during carcinogenesis. (Kawaguchi, Ohno et al. 2000) This tendency has been confirmed in two studies in SCCHN patients, both in Europe and Japan. (Marin, Jost et al. 2000; Tada, Furuuchi et al. 2001) The 72R mutants have been shown to be more efficient in binding to and inhibiting the function of p73, thus limiting the tumour suppressive effect of this p53 family member. Hence the R72 p53 variant may in fact have a gain of function over wild type p53, providing a hypothesis for increased prevalence of R72 mutated p53 in human cancers.

1.16.3 Does codon 72 status affect DNA damage response pathways?

Intrinsic to radiosensitivity in tumour cells is the transduction of DNA damage signals following the formation of DSBs. In normal mammalian cells this is mediated by ATM and CHK2 activation of p53. Where p53 is not functional, other p53 family members may mediate the damage signal leading to cell cycle arrest and/ or apoptosis. If the 72R variant can inhibit the action of both remaining wild type p53 molecules and p63 and p73, then these cells may be more resistant to DNA damage signalling. Thus the effectiveness of radiotherapy may be influenced by the codon 72 status in SCCHN.

1.17 Telomerase inhibitors in combination therapy.

The principle of targeting the telomere addresses the immortality of the cancer cell, a feature which allows specificity of therapeutic attack. In principle any other cancer cell

killing therapy resulting in large scale tumour cell death, would require any residual tumour to then regrow from a small number of cells. All such therapies would therefore be expected to be synergistic with telomerase inhibitors, as more cell divisions would be required and therefore telomeres would be much shorter to achieve any given tumour mass.

A specific approach in the long term might be to combine telomerase inhibitors with new drugs that do not damage DNA and thus do not select for potentially undesirable phenotypes such as deficiency of mismatch repair pathways and, as a consequence, ALT.

One such class of drugs is the angiogenesis inhibitors. Several clinical trials of these agents are underway, both in combination with conventional therapy and alone. Strategies used to inhibit tumour angiogenesis fall broadly into three categories: interference with angiogenic ligands, receptors or downstream signalling; upregulation or delivery of endogenous inhibitors and direct targeting of the tumour vasculature. (Carmeliet and Jain 2000) Anti-angiogenic strategies generally are directed at targets which are features of immature blood vessels and this aspect lends a specificity to this anti-cancer treatment, mature native vessels being relatively spared. One recent study where inhibition of vascular endothelial growth factor-2 (VEGF-2) was investigated showed a reversal of resistance to radiotherapy in two radio-resistant tumour types. (Geng, Donnelly et al. 2001) There are however limitations of pre-clinical trials which mean that caution is needed. Subcutaneous human tumour xenografts in mice may be less robust than human tumours *in vitro* and the endpoint commonly used in preclinical studies is tumour regression rather elimination. With tumour progression a wider array of angiogenic molecules is produced. Hence any therapy directed against a single agent e.g. vascular endothelial growth factor (VEGF) may produce selection of cells expressing an alternative molecule e.g. interleukin 8. Agents against multiple targets used synchronously may be the most effective strategy. Further, current curative therapy for SCCHN involves ablative surgery to remove disease in combination with reconstruction of the affected part. This requires substantial wound

healing, which will involve the immature angiogenesis ligands targeted by antiangiogenesis therapies, albeit at physiological levels. This fact may necessitate a delay in institution of this form of therapy after surgery. It is however common clinical practice to delay for six weeks post-operatively prior to instituting ionising radiation and so the clinical impact of such delay may be small.

The available evidence suggests that angiogenesis inhibitors can limit the expansion of tumours but do not kill the tumour completely (Figure 1-7b). The combined use of angiogenesis inhibitors and radiation is more effective than either alone in human xenografts (Geng, Donnelly et al. 2001) so if sufficient targeting can be applied to tumours greater success might be expected with angiogenesis factor inhibitors in the future. Telomerase inhibitors are complementary to angiogenesis inhibitors in that they kill the tumour very efficiently but generally would not be expected to limit its size at first (Figure 1-7a). Thus the combined use of telomerase and angiogenesis factor inhibitors (see comment by Shay (Randal 2001)) may provide optimal tumour kill in both the immediate and then intermediate treatment phases and in the long term may help prevent the growth of residual tumour following therapy (Figure 1-7c).

The use of angiogenesis and telomerase inhibitors is envisaged to be particularly useful in preventing the growth of occult micro metastases which is a common reason for the relapse of patients with solid tumours.

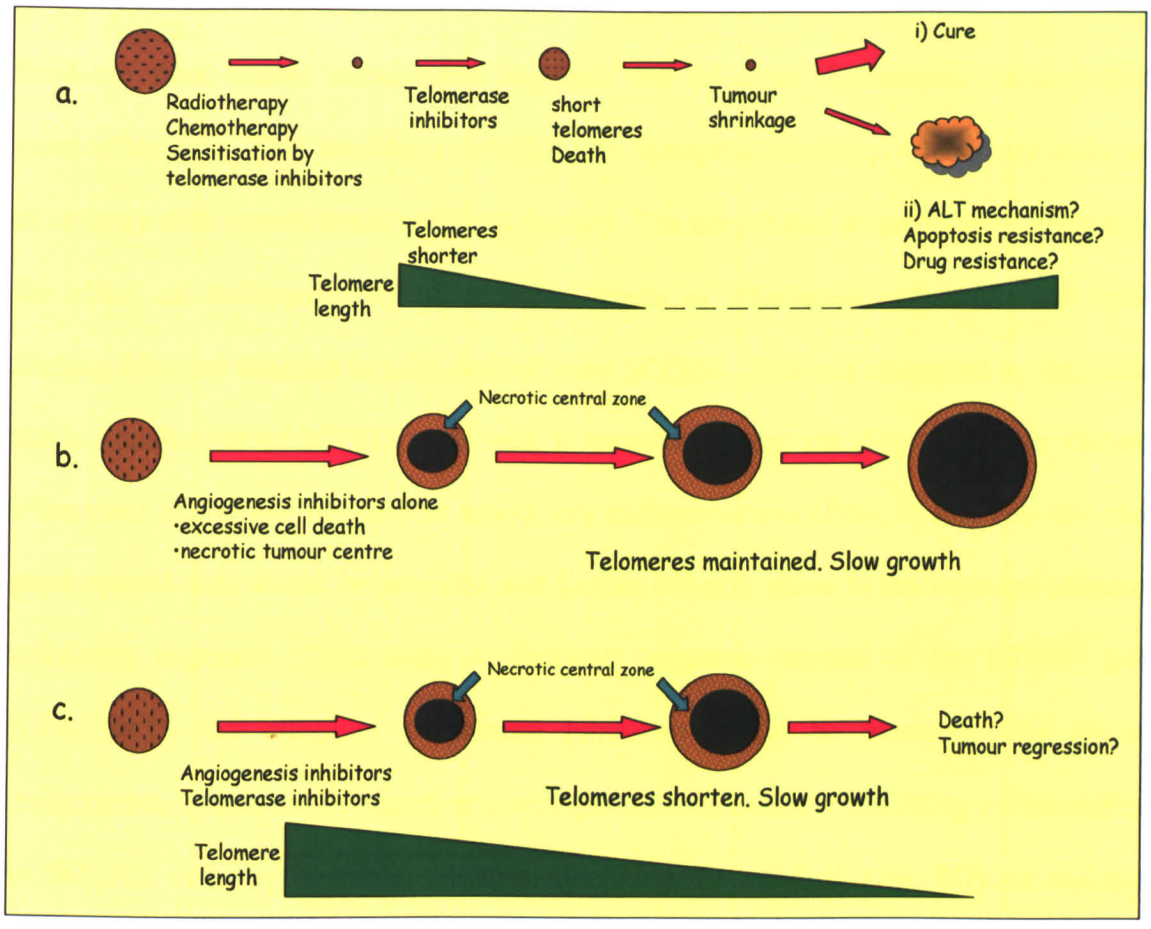


Figure 1-7. Telomerase Inhibitors in Combination therapy

a) This figure summarises the manner in which conventional and telomerase inhibitor therapies might be combined in the future. The possibility of activation of the ALT pathway during prolonged growth under telomerase inhibition is also considered. Following shrinkage by radiotherapy, the tumour would grow for a variable period in the presence of a telomerase inhibitor until the gradual attrition of the telomeres provokes apoptosis and results in considerable cell death (i). It is still debatable whether ALT or other resistance mechanisms might contribute to relapse at this point (ii).

b) Here the limitation of the use of angiogenesis inhibitors alone is illustrated. The inhibitors slow the growth of the tumour by denying the more central cells a blood supply. However, the tumour expands due to the viable outer rim.

c) The same as in (b) but in this situation, the slow growing tumour undergoes telomeric attrition during the multiple divisions required to replace the cells dying in the necrotic centre. The tumour cells are then proposed to die as a consequence of telomere dysfunction. This strategy has the advantage of using two complementary and very specific novel approaches to cancer treatment.

1.18 Aims

Evidence from mouse experiments suggests that telomere dysfunction radiosensitises mammalian cells. This provides a potential new therapeutic strategy, which may make use of synergy with conventional radiation therapy. The aim of this project was to investigate the effect on radiosensitivity of changing levels of telomerase expression and hence altering telomere function in cells derived from SCCHN. This was attempted by first using stable transfection of SCCHN cells with a retroviral vector carrying hTERT to enhance telomerase expression in order to assess any radioprotective effect. If this was the case, good control data would be provided and further strength given to the telomere radiation resistance argument. Then using a dominant negative version of the hTERT gene (DNhTERT), the effect of telomerase inhibition would be assessed. The reverse transcriptase inhibitor AZT was then investigated as a telomerase inhibiting radiosensitiser in SCCHN cells by assessing changes in clonogenic survival after 2Gy of radiation assessed.

The precise role of the p53 protein is not clear, but evidence would suggest that wild type protein is not required for telomere dysfunction- or radiation-induced cell death. The R72 variant is clearly selected for in human cancer. The role played by this variant in the setting of radiotherapy and any impact on cellular radiosensitivity is not clear and was assessed in a panel of SCCHN cell lines. Further information examining any correlation between telomere function (assessed by anaphase bridge index [ABI]) and SF2 and SF4 was obtained.

In order to gain insight into changes in telomere function with SCCHN tumour progression, analysis of ABI in primary tumours and then in recurrent tumours in patients with SCCHN was carried out.

If telomerase inhibition could be shown to enhance radiation induced cell kill then this would strengthen the argument that this may be a valid new therapeutic avenue in SCCHN.

The broader assessment of ABI and SF2 and SF4 may give indication as to whether this method of telomere function evaluation correlates with radiosensitivity. Further, any influence of the R72 polymorphism on SF2 and SF4 was assessed. Should ABI fall with tumour progression then we might predict a more limited response in late stage SCCHN in comparison to those cancers diagnosed and managed earlier in the disease process.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Chemicals

Solutions and buffers were prepared in and where necessary dissolved or diluted using dH_2O from a Millipore MilliRO 15 system.

Supplier	Chemicals
Agar Scientific Ltd., 66A Cambridge Road, Stanstead, Essex, CM24 BDA, UK	Neutral buffered formalin
Amersham International Little Chalfont, UK	γ^{32}P- ATP 3000Ci/mmol, 10mCi/ml
James Burrough (F.A.D.)Ltd 70 Eastways Industrial Park, Witham, Essex, CM8 3YE, UK	Ethanol
Fisher Scientific UK Bishop Meadow Road Leicestershire, LE11 5RG Loughborough UK	Sodium chloride Sodium acetate isopropanol
Hughes and Hughes Unit 1f Lowmoor Ind Estate, Tonedale, Wellington, Somerset	Histomount
Sigma Chemical Company Fancy road, Poole, Dorset, UK	Acetone Bicinchoninic acid Bromophenol blue Copper sulphate EDTA Ethidium bromide Giemsa stain Glycerol Rhodamine B SDS TEMED (N,N,N',N'- Tetramethyl-ethylenediamine Xylene cyanol

Surgipath Venture park, Stirling way, Bretton, Peterborough UK	Haematoxylin Eosin Histoclear Scott's Tap Water

2.1.2 Enzymes

Supplier	Enzyme
Advanced Biotechnologies, Units B1-B2 Longmead Business Centre, Blenheim road, Epson, Surrey UK	Taq polymerase T4 polynucleotide kinase
Gibco BRL Life technologies, Paisley, UK	EcoR 1 Sal 1 Nru 1 Cla 1
Sigma Chemical Company Fancy road, Poole, Dorset, UK	Proteinase K

2.1.3 General plasticware

Supplier	Material
Becton Dickinson Labware Plymouth, UK	Falcon tubes
Bibby-Sterilin Stone,UK	5ml Bijous; 20ml universals
Elkay Galway, Eire	Microcentrifuge tubes Pipette tips
Greiner Labortechnik Stonehouse, UK	Filter pipette tips

2.1.4 Electrophoresis gels

Supplier	Material
Gibco BRL Life technologies, Paisley, UK	Agarose, electrophoresis grade

Severn Biotech Ltd Unit 2, Park Lane, Kidderminster, Worcester, UK	Acrylamide 40% w/v

2.1.5 Paper and X-ray film

Supplier	Material
Whatman International Maidston, UK	3MM paper
H A West, Clydebank, Glasgow, UK	Fuji Super RX medical X-ray film

2.1.6. Microbial host, media and supplies

Supplier	Material
Bibby – Sterilin, Stone, UK	Tryptone Yeast extract
Difco, Becton Dickinson Plymouth, UK	Petri dishes
Gibco BRL Life technologies Paisley, UK	Competent *E. Coli* DH5α cells
Sigma Chemical Company Poole, Dorset, UK	Ampicillin

2.1.7. Drug resistance plasmids

pBabe puro (Hahn, Stewart et al. 1999) was obtained from Dr E K Parkinson at the CRUK Beatson Institute for Cancer Research. The map for this plasmid is shown in figure 2.1 pBabe puro TERT and pBabe puro DNTERT (Hahn, Stewart et al. 1999)were a gift from Professor R. A. Weinberg, Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.

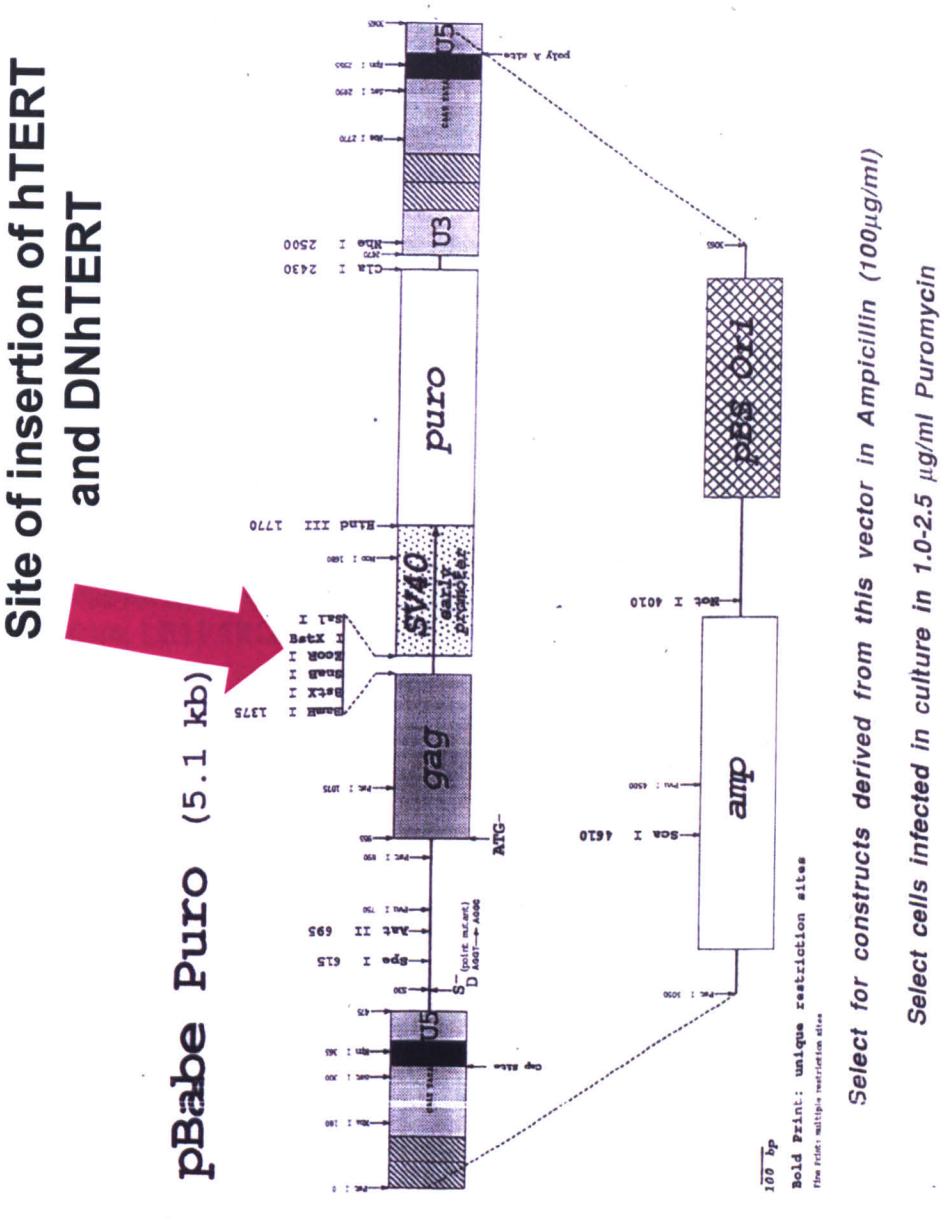


Figure 2-1. Diagram illustrating components of the pBabepuro plasmid

2.1.8 Cell culture media

Supplier	Material
A/S Nunc Botolph Claydon, UK	1ml cryotubes
Autogen Bioclear Holly Ditch Farm, Mile Elm, Calne, Wiltshire, UK	Fetal Bovine Serum
Becton Dickinson Labware Plymouth, UK	Falcon tissue culture dishes;
Fisher Scientific UK Bishop Meadow Road Leicestershire, LE11 5RG Loughborough UK	Dimethylsulfoxide
Gibco BRL Life Technologies Paisley UK	DMEM Hepes buffer L-Glutamine
Sigma Chemical Company Fancy road, Poole, Dorset, UK	Benzylpenicillin Streptomycin Hydrocortisone Bovine donor calf serum
Worthington Chemical Corporation Reading, UK	Trypsin

2.1.9. Supplies for specialised cell culture techniques

Supplier	Material
Gibco BRL Life Technologies Paisley UK	Lipofectamine
A/S Nunc Botolph Claydon, UK	Chamber slides
Sigma Chemical Company Fancy road, Poole, Dorset, UK	Polybrene Optimem Lipofectamine
Merck (WDR International) Poole, Dorset, UK	Acrodisc syringe filters; 0.2μm, 0.45μm

2.1.10. Cell lines

The following cells are SCCHN lines derived from *in vivo* tumours. (Edington, Loughran et al. 1995) These were obtained from Dr E K Parkinson at the CRUK Beatson Institute for Cancer Research;

Table 3. Keratinocytes supplied by Dr E K Parkinson.

Keratinocyte line	Site of origin	Tumour TNM stage	Proliferative fate
BICR 3	Alveolus	$T_2N_0M_0$	immortal
BICR 6	Hypopharynx	$T_4N_1M_0$	immortal
BICR 7	Tongue	$T_4N_{2c}M_0$	Mortal; Crisis (PDL=42)
BICR 10	Buccal Mucosa	Recurrent	immortal
BICR 16	Tongue	Recurrent	immortal
BICR 31	Tongue	$T_4N_{2B}M_0$	immortal
BICR 56	Tongue	$T_4N_1M_0$	immortal
BICR 63	Tongue	$T_2N_{2B}M_0$	immortal
BICR 68	Tongue	$T_4N_0M_0$	immortal
BICR 78	Alveolus	$T_4N_1M_0$	immortal
BICR 82	Maxilla	Recurrent	immortal

The following cell lines were also utilized in this thesis.

Table 4. Other cell lines.

Cell line	Cell type	Source
Swiss 3T3	Mouse embryonic fibroblasts	ATCC 12301 Parklawn Drive Rockville Maryland, 20852 USA
NIH 3T3	Mouse embryonic fibroblasts	ATCC 12301 Parklawn Drive Rockville Maryland, 20852 USA
HFF	Human foreskin fibroblasts	Dr E K Parkinson, BICR (Method described in (Burns, Baird et al. 1993))
Phoenix A	Retroviral producer line	Dr G P Nolan, Stanford University Medical Centre, San Francisco, California USA
DH5α	Modified E. Coli bacteria	Gibco BRL Life Technologies Paisley, UK
GM847	SV40 immortalised human fibroblasts (ALT cell line)	Dr Olivia Pereira-Smith Baylor College of Medicine, Waco, Texas, USA.
SCC 4 SCC 9 SCC 15 SCC 25	Squamous cell carcinoma (all of lingual mucosa origin)	ATCC 12301 Parklawn Drive Rockville Maryland, 20852 USA

2.1.11. Radiation source

An Alcyon 2 radiotherapy machine, located in the CRUK Beatson Institute for Cancer Research was used as the ionising radiation source in experiments detailed in this thesis. This is a ^{60}Co source, providing gamma irradiation.

2.1.12. Molecular Biology kits.

Table 5. Molecular Biology Kits

Supplier	Kit
Qiagen Ltd Crawley, West Sussex UK	QIAGEN plasmid midi kit
Intergen Company Oxford UK	TRAPEZE Telomerase detection kit

2.2 Methods.

2.2.1 Cell culture techniques

2.2.1.1. Culture of cell lines

All keratinocyte cell lines and clones were cultured in DMEM supplemented with 10% (v/v) FBS, 2mM L-glutamine, 20mM HEPES buffer, 50U/ml penicillin, 20ng/ml streptomycin, and 0.4μg/μl hydrocortisone (10H medium).

Human foreskin fibroblasts and GM847 fibroblasts were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 20mM HEPES buffer, 50U/ml penicillin and 20ng/ml streptomycin (10C medium). When experiments were carried out comparing both HFFs and GM847 cells to malignant keratinocytes, all cells were grown in keratinocyte medium as described above. Phoenix A cells, Swiss 3T3 cells and NIH 3T3 cells were grown in DMEM supplemented with 10% DCS, 2mM L-glutamine, 20mM HEPES buffer, 50U/ml penicillin and 20ng/ml streptomycin (10C medium). All cell lines were maintained in a humid 37°C, 5% CO_2 incubator.

Prior to trypsinising keratinocyte for experiments or for replating or storing in liquid nitrogen, feeder cells were removed by exposure of the cell layer to 0.02% EDTA in PBS for 30 seconds, followed by vigorous pipetting. Remaining keratinocytes were washed with PBS and then trypsinised in a humid 37°C, 5% CO_2 incubator for 15 minutes using equal volumes of 0.02% EDTA in PBS and 0.1% trypsin.

Cryopreserved cell stocks were prepared from early passage cell lines and clones as follows. Cells were maintained in culture until they reached approximately 75% confluence. They were then trypsinised, neutralised with normal growth medium and pelleted. The cell pellet was resuspended in freezing medium (10% DMSO in normal growth medium) to a density of 1 x 10^6cells/ml, and the suspension transferred to

cryotubes (1ml per vial). The cryotubes were wrapped in cotton wool and placed in a -70°C freezer overnight to slow freeze and the following day were immersed in liquid nitrogen for long term storage. Cryotubes were recovered from liquid nitrogen by immediate immersion in a large volume of water at 37°C. The cell suspension was then made up to a volume of 10ml with normal growth medium and the cells pelleted. Cells were resuspended and plated at a density of 0.5-1.0 x 10^6 cells per 10cm dish.

2.2.1.2. Transfection of Phoenix A cells

Cells were cultured in 9cm plates until approximately 50-80% confluent. Two solutions were prepared; one containing 20μg of plasmid DNA in 650μl of Optimem serum free medium and one containing 34μg of lipofectamine reagent and 642μl of Optimem. These were then combined and allowed to stand at room temperature for 30 minutes to allow liposomal complexes to form. The Phoenix A cells were washed once in PBS. Optimem was added to each of the transfection solutions to a volume of 5mls. These solutions were pipetted onto the producer cells and incubated at 37°C in humid 5%CO2 for 5 hours. A control plate of cells was treated with lipofectamine reagent and Optimem serum free medium only. Following incubation 7mls of growth medium containing twice normal concentration of serum was added, without removing the transfection mixture. The plates were incubated overnight (approximately 18 hours) at 37°C before the transfection mixture was removed and replaced with complete growth medium. The cells were incubated for a further 24 hours at which point retrovirus containing supernatant was harvested.

2.2.1.3. Selection determination

Kill curves were used to determine the appropriate level of antibiotic selection to use in transfections. Cells were plated at 10^5 per 6 cm plate and placed under selection media after 48 hours. Thereafter selection media was changed twice weekly. Complete cell death after two weeks was taken to be the appropriate antibiotic selection. Appropriate cell line selection determination values are shown in table 2.1

Table 6. Puromycin concentration for selection in feeder cells and keratinocytes

Cell line	Puromycin concentration
NIH3T3	2μg/ml
Swiss 3T3	2μg/ml
BICR 6	1μg/ml

2.2.1.4 Viral titre assessment

NIH 3T3 cells were seeded at a density of 10^5 cells per 6 cm dish and cultured at 37°C in humid 5% CO_2 for 24 hours. Serial dilutions of virus were produced by adding 100μl of virus containing supernatant to growth medium with 4μg/ml polybrene added to a volume of 4mls. This was diluted to produce concentrations of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Plates were infected with 1.5mls of appropriate dilutions and incubated for four hours. Growth medium was added to a final volume of 5ml and the plates incubated overnight. Plates were then medium changed and after a further 24 hours in culture selection commenced by the addition of 2μg/ ml puromycin. Selection was performed for ten days followed by staining with Giemsa and the viral titre calculated.

2.2.1.5. Viral Infection of Swiss 3T3 cells, HFFs and GM847 cells.

Puromycin resistant Swiss 3T3 feeder cells were generated by infecting with retrovirus produced in Phoenix A amphotropic producer cells. This virus included the pBabe puro plasmid and hence conferred puromycin resistance. Swiss 3T3 cells were plated at a density of 5 x 10^5 cells per 9cm plate and cultured in humid 5%CO_2 at 37°C for 72 hours. At this point 2mls of neat retrovirus containing supernatant was applied to the cell layer with 8mg/ml of Polybrene and incubated for four hours. Duplicate mock plates had

Polybrene containing medium only added during the incubation period. Following incubation growth medium was changed for fresh 10C and the cells allowed to recover overnight. Cells were then split by trypsinisation and seeded at a density of 3 x 10^5 on 9cm plates. After a further 72 hours cells were placed under selection with medium containing 2μg/ml puromycin. Puromycin resistant colonies were then pooled and replated onto 9cm plates. Further pools of colonies were stored in liquid nitrogen for further use. The same method was used to generate HFFs and GM847 fibroblasts infected with virus carrying pBabe puro, pBabepurohTert and pBabepuroDNhTert.

2.2.1.6. Viral Infection of keratinocytes.

Cells to be infected were seeded in duplicate at a density of 5 x 10^5 cells per 6cm plate with 3 x 10^5 lethally irradiated puromycin resistant feeder cells 72 hours prior to infection. Viral supernatant was added with 8mg/ml Polybrene and incubated for four hours. Duplicate mock plates had Polybrene containing medium only added during the incubation period. Following incubation growth medium was changed for fresh 10H and the cells allowed to recover overnight. Cells were then split by trypsinisation and seeded at a density of 3 x 10^5 on 9cm plates with 9 x 10^5 puromycin resistant irradiated Swiss 3T3 cells. After a further 72 hours cells were placed under selection with medium containing 2μg/ml puromycin. Puromycin resistant colonies were then ring cloned into individual 6cm plates and pools of colonies stored in liquid nitrogen for further use.

2.2.1.7. Determination of AZT dosage.

BICR 6 cells were cultured in 9 well plates in duplicate wells on a feeder layer of lethally irradiated 3T3 cells. 10^5 cells per well were allowed to grow in culture for 48 hours prior to the introduction of AZT at the following concentrations; control, 50μM, 100μM, 200μM, 400μM, 800μM, 1mM, 1.4mM and 2mM.

The maximum dosage which still allowed cells to reach confluence was 100μM AZT. The range for telomerase inhibition and radiosensitivity testing was therefore selected as control, 50μM AZT and 100μM AZT.

2.2.1.8. Rhodamine Stain for Keratinocytes

Medium was aspirated from plates containing adherent cells and these were washed with PBS. 10% formaldehyde was then overlaid for 15 mins. This was aspirated and the cells then washed again with PBS. Rhodamine B stain (1%) was then overlaid for 30mins. Cells were then rinsed in tap water until all excess stain was removed, air dried and then observed under a low power microscope and colonies counted.

2.2.1.9. Giemsa Stain for 3T3, HFF and GM847 cells.

Medium was aspirated from plates containing adherent cells and these were washed with PBS. 10% formaldehyde was then overlaid for 15 mins. This was aspirated and the cells then washed again with PBS. Giemsa stain (10%) was then overlaid for 30mins. Cells were then rinsed in tap water until all excess stain was removed, air dried and then observed under a low power microscope and colonies counted.

2.2.1.10 Determination of population doublings.

The number of population doublings achieved in the dominant negative hTERT clones was calculated at each passage according to the formula of Paul *et al.*, 1975;

$$PD = (\log N - \log N_0) \times 3.32$$

Where N_0 = initial cell number

N = final cell number

Equation 1. Formula for calculation of population doublings in cultured cells

2.2.1.11. Culture of cells for Anaphase Bridge Scoring.

Keratinocytes were plated on single well chamber slides at a density of 8 x 10^4 with X lethally irradiated Swiss 3T3 cells. These were culture at 37°C in 5% humid CO_2 until reaching 50 – 70% confluence. Growth medium was aspirated, cells washed with PBS and placed in acetone at 0°C for 15 minutes. These were then post-fixed in neutral buffered formalin for 5 mins, washed in water and stained with haematoxylin for one minute. After a further rinse in water the slides were placed in Scott's tap water for one minute. Slides were immersed in eosin for 20 seconds, rinsed once more in water and dehydrated in graded alcohols before placing in Histoclear for one minute. These were then mounted using silicon glass cover slips and histomount.

2.2.2 Microbiological techniques.

2.2.2.1. Transformation of bacteria with plasmid DNA

Competent *E. Coli* (DH5α) were thawed on ice and aliquots transferred to prechilled 1.5ml screw cap microcentrifuge tubes. Approximately 10ng of plasmid DNA was added and gently mixed with the cells by stirring with a pipette tip. After incubation on ice for 30 mins, cells were heat shocked at 42°C for 45s and then placed on ice for a further 2 mins. 400μl of SOC medium (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 20mM glucose, 10mM NaCl and 10 mM $MgCl_2$) was then added to the mixture and the cells were incubated at 37°C for one hour in an orbital shaker at 240 rpm. After this time cells were pelleted by pulsing in a microcentrifuge. 450μl of supernatant was discarded and the cells resuspended in the remaining 50μl. Cells were then spread on LB medium plates containing 1.5% (w/v) agar supplemented with the appropriate antibiotic (ampicillin). Plates were incubated in the inverted position at 37°C, and colonies transferred into LB medium the following day.

For the preservation of bacterial stocks, a 0.5ml aliquot of overnight culture in LB medium (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl, pH 7.0) was mixed with an equivalent volume of 50% (w/v) glycerol, chilled on ice and then stored at -70°C in plastic cryotubes.

2.2.2.2. Plasmid DNA Extraction

Plasmid DNA was extracted using the QIAGEN Midi kit. A single colony was picked from a selective plate (see section 2.2.2.1) and used to inoculate a 5ml starter culture of LB medium containing 100μg/ml ampicillin. The culture was incubated for 8 hours at 37°C in an orbital shaker at 240 rpm , diluted 1/1000 into selective LB medium and incubated for a further 16 hours at 37°C in an orbital shaker at 240 rpm. The bacterial cells were harvested by centrifugation at 6000 x g for 15 mins followed by removal of supernatant. These were then resuspended in 10ml of P1 buffer. An equal volume of P2 buffer was added and these gently mixed by inversion, followed by incubation at room temperature for 5 mins. 10mls of buffer P3 was added and the solution then incubated for a further 20 mins. This was then centrifuged at 20,000 x g for 30 mins at 4°C to separate plasmid DNA from cell debris. The supernatant from this spin was removed and promptly respun at 20,000 x g for 15 mins at 4°C. A QIAGEN 500 tip was equilibrated by running 10mls of QBT buffer through it by gravity flow. The supernatant was passed through the equilibrated tip under gravity flow, passing through plasmid DNA retaining resin. The QIAGEN tip was washed twice with 30ml of QC buffer. DNA was eluted from the resin using 15ml of QF buffer and collected in a 30ml tube. This was then precipitated by adding 0.7 volumes of isopropanol to the eluted DNA. This mixture was then centrifuged at 15,000 x g for 30 mins at 4°C and the supernatant decanted leaving the DNA pellet in the tube. This was washed with 5mls of 70% ethanol and centrifuged at 15,000 x g for 10 mins at 4°C followed by careful decanting of supernatant, leaving the pellet undisturbed. This was then air-dried for 10 mins and then redissolved in TE buffer, pH 8.0. Plasmid DNA concentration was determined as described for genomic DNA.

2.2.3. Irradiation of cells in culture.

2.2.3.1. Irradiation of feeder layer Swiss 3T3 cells.

Cells were trypsinised into suspension, centrifuged and neutralised in normal medium. Cell density was calculated using a haemocytometer. Universals containing these cells were maintained on ice and transported to the radiation suite. Universals were placed in the radiation source at a distance of 0 cm under an intensifying screen. Flasks containing water were placed around the specimen universals in order to achieve uniform scatter of ionising radiation throughout the field. Exposure to 60Gy provides feeder 3T3 cells which support keratinocyte growth without 3T3 cell replication.

2.2.3.2. Irradiation of keratinocytes.

Feeder layer cells were removed by as described above and keratinocytes trypsinised, neutralised in normal medium, centrifuged at 1000 rpm at 4°C for 5 minutes and then resuspended in fresh medium. Cell density was calculated using a haemocytometer and appropriate dilutions prepared to obtain 2 x 10^5 cells in 5mls of growth medium. These were maintained on ice in universal tubes and transported to the radiation suite. Universals were placed in the radiation source at a distance of 100cm under an intensifying screen. Flasks containing water were placed around the specimen universals in order to achieve uniform scatter of ionising radiation throughout the field. Cells were then plated at an appropriate density to obtain between 50-200 colonies per plate, this density having been calculated from earlier experimental data for each cell line studied.

2.2.4. Extraction, quantification and sequencing of genomic DNA.

2.2.4.1. Extraction of genomic DNA from human keratinocytes.

Genomic DNA was prepared from malignant human keratinocytes using the method of (Laird, Zijderveld et al. 1991). Feeder layer fibroblasts were removed as described above and cells harvested by trypsinisation and pelleted by centrifugation (5 mins at 4°C at 1000 rpm) in a universal tube. Cells were resuspended in PBS and transferred to a

microcentrifuge tube before brief centrifuging . These were then resuspended and simultaneously lysed in lysis buffer (100mM Tris.HCl pH 8.5, 5mM EDTA, 0.2% (w/v) SDS, 200mM NaCl and 100μg/ml proteinase K) and incubated at 37°C with constant agitation. DNA was precipitated by addition of an equal volume of isopropanol, with gentle mixing until all viscosity was gone. The aggregated precipitate was removed by lifting from the solution with a sterile custom made loop (made by heating a glass Pasteur pipette tip in a Bunsen flame). Excess liquid was dabbed off and the DNA rinsed in 70% (v/v) ethanol. After being briefly allowed to air-dry, the DNA was dispersed into at least 0.5 ml of TE (10mM Tris.HCL and 1mM EDTA pH 8.0) and maintained at 37°Cwith agitation until completely dissolved.

2.2.4.2. Quantification of DNA.

DNA was quantified by spectrophotometric determination of it UV light absorbancy. 5μl of sample was added to 495μl of deionised water and the absorbency of the solution measured at 260nm and 280nm in a disposable plastic cuvette, using dH_2O as a blank. The concentration of the solution was calculated using de Beer's law on the basis that an optical density of 1.0 at 260nm corresponds to a concentration of 50μg/ml for double stranded DNA and 33μg/ml for single stranded oligonucleotides. Pure preparations of DNA and RNA have a ratio of A_{260}/A_{280} readings between 1.8 and 2.0.

2.2.4.3. Sequencing of p53 codon 72.

Extracted and quantified genomic DNA was analysed for codon 72 argenine or proline status by the CRUK BICR DNA sequencing service.

2.2.5. Assessment of Anaphase Bridge Indices.

2.2.5.1. Anaphase bridge scoring for culture slides.

Mitotic figures in late anaphase were located at x200 magnification and then scored at x400 under an Axioskop microscope in the CRUK BICR. At least 30 anaphase mitoses were scored for each cell line assessed by the author and also independently by Dr E K Parkinson, the vernier number for each being recorded manually. An anaphase bridge figure is defined as a mitosis in late anaphase where trailing chromatin from daughter chromosomes extends more than 2/3 of the distance across between anaphase plates. There must be sufficient distance between the plates to allow assessment and no evidence of a cleavage furrow heralding the onset of telophase present.

2.2.5.2. Anaphase bridge scoring for archival primary and recurrent SCCHN tumours and BCCa tumours.

The archive of oral squamous cell carcinoma specimens held in the Oral Pathology department of Glasgow Dental Hospital and School was analysed by Professor D G MacDonald in order to locate primary and recurrent tumour specimens from the same site in the same individual patients. Specimens were coded and the observer blinded as to the identity of each slide. For each patient a disease free interval of at least six months following treatment and prior to the clinical emergence of recurrent carcinoma was required for inclusion in the study. For each tumour specimen at least 30 appropriate anaphase mitotic figures were scored. Where an insufficient number of anaphases were found in the archival tumour slides, fresh sections were prepared from archival tumour blocks.

H & E stained slides were analysed on an Olympus BX40 light microscope in the Oral Pathology department of Glasgow Dental Hospital and School. Anaphases were recorded as either normal or bridged and the vernier number manually recorded.

2.2.6 Telomere Repeat Amplification Protocol.

2.2.6.1. Preparation of cell extracts.

Cells to be assayed had the feeder layer removed and were trypsinised and counted as detailed above. Centrifuging for five minutes at 1000rpm was followed by washing once with PBS. Cells were then pelleted again by spinning at 12,000 x g in a microfuge at 4°C for five minutes and the PBS carefully removed. Cells were then resuspended (10^5 – 10^6 cells) in 200μl of 1 x CHAPS lysis buffer. These were incubated on ice for 30 minutes and the supernatant then retained (as 2 x 10μl aliquots), frozen on dry ice and then stored at -80°C. Extracts can be stored for up to one year.

2.2.6.2. Bicinchoninic acid (BCA) protein assay.

Bovine serum albumin solution was prepared in dH_2O to produce a stock of 2mg/ml solution. From this, stocks of BSA solution in CHAPS lysis buffer were prepared in a range of concentrations from 0.08mg/ml up to 2mg/ml. These were placed in wells in a microreader plate and 200μl of bicinchoninic acid solution (9.8ml of BCA combined with 200μl of $CuSO_4$) added to each well. 10μl aliquots of each whole cell protein extract were placed in duplicate wells and BCA added as previously. The plate was then incubated at 37°C in a hot room for 45 minutes. Light absorbencies were read at 570nm using a Dynatech MR7000 spectrophotometer to produce a standard curve for protein and a reading for each protein sample was estimated from this.

2.2.6.3. Preparation of controls.

Heat inactivated controls were generated for each cell line tested by treating 4μl of protein extract for 10 minutes in a hot block at 85°C. Two microlitres of this heat inactivated extract were subsequently used in the assay. Primer/ dimer/ PCR contamination control was provided by use of 2μl of 1 x CHAPS lysis buffer in one lane. A telomerase positive quantitation control template (TSR8) provided in the TRAP kit was run in another lane.

2.2.6.4. Radioactive end labelling of TS primer.

For twelve assays, γ^{32} P-ATP (4μl) was combined with TS primer (16μl), 10x kinase buffer (3.2μl) and T4 PNKinase (10unit/ μl; 1μl) and 7.8μl of dH_2O. These were incubated for 20mins at 37°C and then the enzyme heat inactivated at 85°C for five minutes. Two microlitres were used per TRAP assay reaction.

2.2.6.5. Preparation of master mix and reaction tubes.

In a separate room in the laboratory, the following components were combined using Gilson pipettes kept solely for PCR experiments; 10xTRAP reaction buffer (70µl), 50xdNTP mix (14µl), TRAP primer mix (14µl), dH_2O (540.4µl) and Taq polymerase (5units/µl; 5.6µl). Radiolabelled TS primer was then added (2μl per assay). This mastermix was then added to thin-walled reaction tubes to a final volume of 50μl per tube.

2.2.6.6. PCR amplification.

This was carried out using an Applied Biosystems Gene Amp PCR System 9600 machine in two steps. The first was 30°C for 30 mins during which time telomerase mediated formation of radio end labelled DNA repeats occurred and the second was 30 cycles of 95°C for 30s and 59°C for 30s during which taq polymerase mediated amplification of reaction products occurred.

2.2.6.7. Polyacrylamide gel electrophoresis and data analysis.

Loading dye was added to each reaction tube and 25 μl of the sample run on a 10% non-denaturing PAGE gel with 0.5x TBE running buffer. Heat treated and non-heat treated samples were loaded in alternate lanes. The negative control lane was added first and the positive control lane last to avoid carry-over contamination. This was run at 400 volts for 1.5 hours until the Xylene cyanol was 70-75% down the gel and the Bromophenol blue had just run off the bottom of the gel. The gel was then fixed for 20 minutes in a solution of methanol, sodium acetate and sodium chloride and then dried on a gel drier. Reaction products were then visualised on the BioRad molecular Phosphoimager and the telomerase

products generated calculated by comparing the ladder of products to the 36bp internal standard using Quant One imaging software. An autoradiograph was also generated by exposing the dried gel to x-ray film and developing this in a Kodak developer.

2.2.6.8. Semi-Quantification of Telomerase activity.

The signal generated from the area of the gel corresponding to TRAP products was measured in all samples including the non-heat treated (X) and heat treated lanes (X_o) and from the CHAPS lysis buffer control (R_o) and the TSR8 quantitation control (R). The signal was measured from the 36bp internal standard in the non-heat treated samples (C) and the TSR8 quantitation control (C_R). The telomerase products generated were calculated from the following formula;

$$\textbf{TPG (units)} = \frac{\textbf{(X-Xo)/ C}}{\textbf{(R-Ro)/ Cr}} \times 100 \quad \textbf{(where 0.1mole = 1}\mu\textbf{l of TSR8)}$$

TPG = telomerase products generated.

Equation 2. Formula for calculation of telomerase DNA products generated in the TRAP assay.

Chapter 3

Results.

Radioprotection of SCCHN cells *in vitro* with increased expression of telomerase.

3.1 Introduction

A body of evidence now suggests that the telomere is a crucial structure in determining the cellular response to ionising radiation. Yeast evidence shows that double strand break repair proteins (yKu80 and Sir3p) are bound at the telomere and are mobilised from there to the site of DNA damage during cellular repair processes. (Boulton and Jackson 1996; Martin, Laroche et al. 1999; Mills, Sinclair et al. 1999) Telomere length in yeast has been shown to influence response to ionising radiation. Mutations in yeast DNA repair proteins (yKu80, yKu70, Mre11, Rad50, Xrs2, Sir2 and Sir3) alters both telomere length and sensitivity to irradiation.(Haber 1998) In the nematode *Caenorhabditis elegans* mutation of DNA repair mtr-2 causes shortening of the telomeres and enhanced cell kill in response to irradiation. (Ahmed and Hodgkin 2000) In studies of the human genetic disorders Ataxia telangiectasia (AT) and the Nimjegen breakage syndrome (NBS) further relevant evidence has arisen. Patients with AT are highly radiosensitive and are cancer prone. (Taylor, Harnden et al. 1975) The NBS protein is found at telomeres and cells from NBS patients are also radiosensitive. (Lombard and Guarente 2000)In mammalian cells the telomere is a site of DNA DSB repair protein binding and an association has been shown between telomere shortening and ionising radiation sensitivity both in human tissue (McIlrath, Bouffler et al. 2001) and in mice. (Goytisolo, Samper et al. 2000; Wong, Chang et al. 2000). Further relevance of the telomere as a DSB repair protein binding site is shown by the high incidence of telomeric fusions observed in Ku- deficient mice, which are radiosensitive. (Bailey, Meyne et al. 1999)

The upregulation of the telomerase enzyme maintains the telomere at shortened length in human malignant cells. Based on the evidence above, the inhibition of this enzyme, producing further shortening of the telomere in human cancer cells may therefore enhance radiosensitivity. We initially examined the converse, by assessing the effect of increasing telomerase expression in a rare, telomerase deficient cancer cell line and comparing this

effect to that seen in cancer cells already with high telomerase levels. Each of two cell lines had been stably transfected with a retroviral vector carrying either the empty vector pBabeNeo or the hTERT expression vector pBabest2 by Dr K E Gordon, (Gordon, Ireland et al. 2003)who kindly donated these cells. These are detailed below.

3.2 BICR 7 shows a crisis phenotype.

Cell lines derived from *in vivo* SCCHN tumours at the CRUK BICR by Dr E K Parkinson (Edington, Loughran et al. 1995)and the R9 research group show a variety of different telomere lengths and levels of telomerase upregulation. (Gordon, Ireland et al. 2003) The BICR 7 cell line has short telomere restriction fragments (TRFs) on Southern blotting (3Kb) and a relatively low level of telomerase. This is reflected in a high anaphase bridge index (0.55) and a low cloning efficiency (3%) when compared to other similarly derived lines (<0.2 ABI and cloning efficiency of >25%). BICR 7 is in fact mortal, despite having bypassed M0 and M1 as shown by p16 and p53 immunostaining (Gordon, Ireland et al. 2003) The BICR7Tert cells however have a much enhanced expression of telomerase (increased 80 fold) and increased TRF lengths (10Kb). This is in fact a telomere length in excess of that seen in pre-M0 keratinocytes. (Dickson, Hahn et al. 2000) The cloning efficiency of these cells is increased to 28% and the anaphase bridge index falls to 0.07. This evidence therefore shows the reversal of telomere dysfunction with increased telomerase.

3.3 BICR 6

In contrast, the BICR 6 line shows longer TRFs (6.5kb) and higher endogenous levels of telomerase. This cell line grows well in tissue culture and is immortal, with few anaphase bridge figures and a cloning efficiency of 33%. When supraphysiological levels of telomerase are expressed in the BICR6Tert cells, the cloning efficiency, which is already high, is unaffected. Telomere length, however is increased to >10kb, again longer than normal, pre-M0 keratinocytes.

Comparisons between levels of enzyme expression and the effect on telomere length and function is given in table 3.1

Table 7. Telomerase activity and telomere function in BICR6Neo, BICR6Tert, BICR7Neo and BICR7Tert. (Gordon, Ireland et al. 2003)

Cell line	Telomerase catalytic activity (TPG)	Cloning efficiency	TRF length	Anaphase bridge index
BICR6Neo	145.8	33%	6.5kb	0.09
BICR6Tert	320.5	37%	>10kb	0.02
BICR7Neo	1.7	3%	3kb	0.55
BICR7Tert	192	28%	>10kb	0.07

3.4 Radiation survival curves for BICR6Neo, BICR6Tert, BICR7Neo and BICR7Tert.

These cells were used experimentally to examine the influence of the telomerase enzyme on radiosensitivity, affording a contrast between cells with low telomerase expression and many features of the crisis phenotype (BICR 7) and a cell line with stable telomere function and higher telomerase levels (BICR6). The effect of supraphysiological expression of telomerase on radiation survival could then be assessed.

Each cell line was grown in tissue culture as described in materials and methods. Cells in suspension were irradiated with 0,1,2,3,4,5 and 10 Grays of radiation for BICR 6Neo and BICR6Tert and with 0, 2 and 4 Grays for BICR 7. Each assay was calculated with two replicate plates. Survival at these levels of ionising radiation exposure was then assessed by calculating cloning efficiency as described in materials and methods. Each experiment was completed three times and the data pooled.

3.4.1. Radiation survival at 0 – 10 Grays for BICR6Neo vs. BICR6Tert.

Data for BICR6 Neo and for BICR6 Tert is presented graphically in figures 3-1. The scale here is logarithmic. The comparison of data from these two cell lines demonstrates no appreciable effect on radiosensitivity in BICR6 cells when telomerase expression is increased to supraphysiological levels with corresponding changes in TRF length over BICR 6 cells expressing drug resistance vector only.

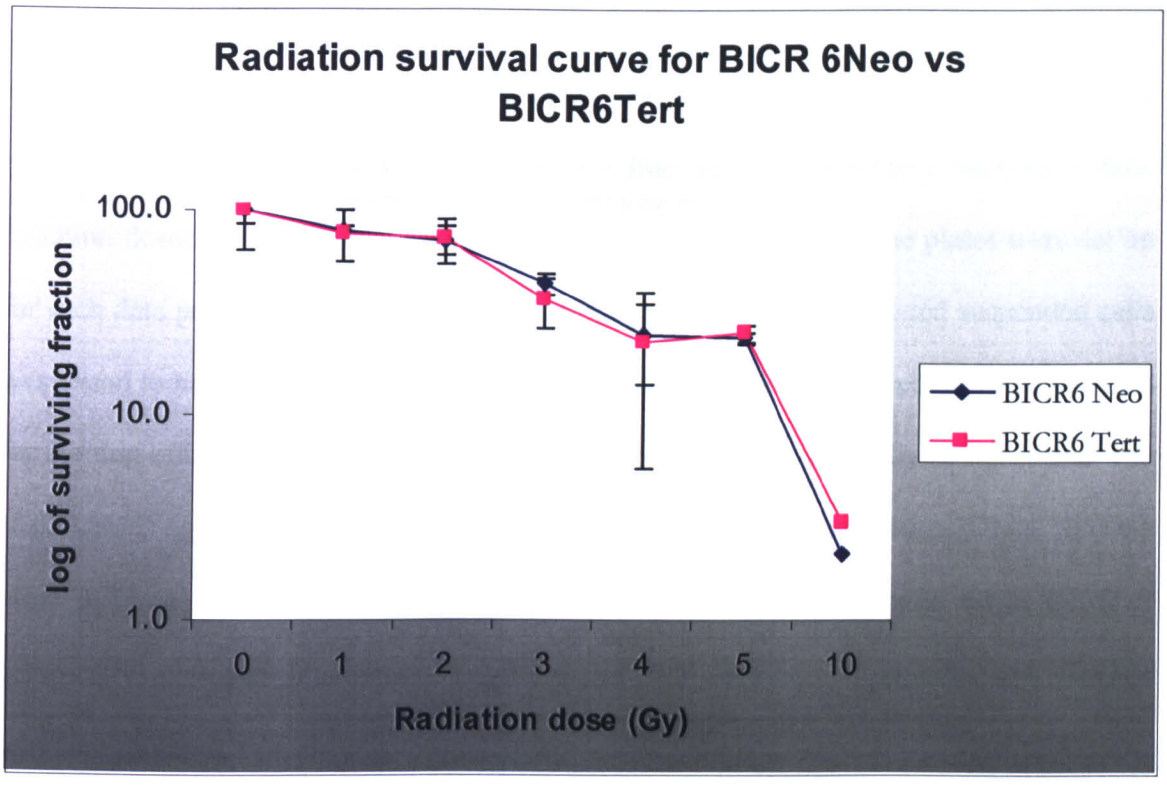


Figure 3-1. Radiation survival curve for BICR6Neo vs. BICR6Tert.

Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Differences are not statistically significant between these cell lines for any ionising radiation dose (Mann-Whitney U-test).

3.4.2. Radiation survival at 0 – 4 Grays for BICR7Neo vs. BICR7Tert.

Early experiments with BICR7Neo indicated very low radiation survival with ionising radiation doses greater than 4 Gy. For this cell line, in order to achieve analysable data, radiation doses of 0, 2 and 4Gy were therefore selected. Two replicate plates were set up for each data point. Additionally, ten fold increases in plating of irradiated suspended cells was found to be appropriate in order to allow the growth, staining and counting of colonies for cloning efficiency calculation. This data is demonstrated graphically in figure 3.2. The scale here is again logarithmic. The combination of data from these two cell lines shows a clear difference in radiosensitivity. There is improved radioresistance when BICR 7 ectopically expresses telomerase, lengthens the TRF restriction fragments and restores telomere function.

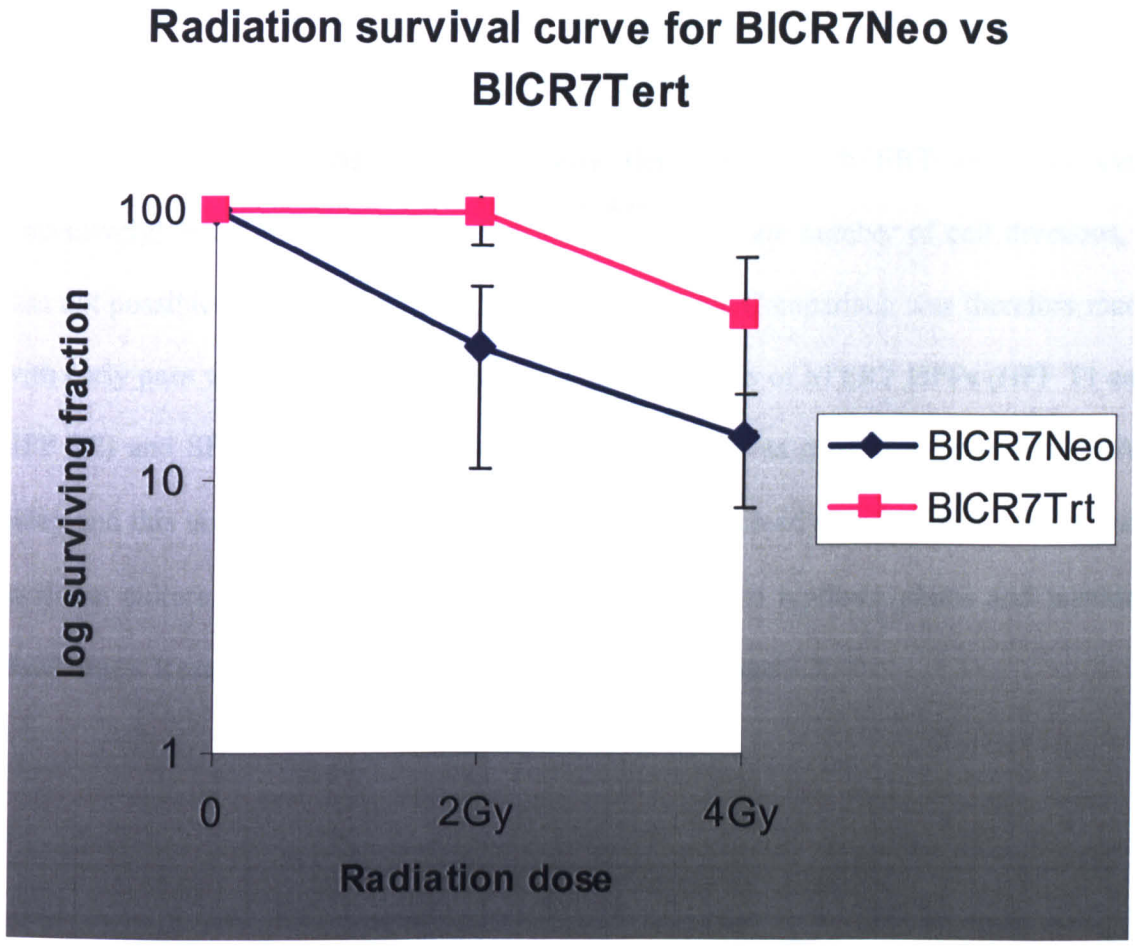


Figure 3-2. Radiation survival for BICR7Neo and BICR7Tert.

Radiation survival for BICR7Neo and BICR7Tert at 0, 2 and 4Gy expressed as survival fraction (%).Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Difference in SF2 and SF4 between drug resistance vector only and hTERT expressing cells are statistically significant ($p<0.01$ in both cases; Mann-Whitney U test).

3.5. Radiosensitivity in HFFs with ectopic expression of telomerase.

Early pass HFF cells were grown in culture as described in chapter 2. These were then transfected with Phoenix A retrovirus carrying the hTERT gene. Clones were selected and pools subjected to 2 Gy of ionising radiation as described. As control cells transfected with DNhTERT and drug resistance vector only virus (pBabeDNhTERT and pBabepuro respectively) failed to undergo cell division for an adequate number of cell divisions, it was not possible to compare these cells to hTERT HFFs. Comparison was therefore made with early pass wild type HFF cells and two separate pools of hTERT HFFs (HFF T1 and HFF T2) and SF2 calculated. Expression of telomerase was confirmed using the TRAP assay and this is shown in figure 3-3. hTERT HFF clones clearly replicated at a higher rate in tissue culture. Experiments were carried out using two replicate plates and repeated three times. Results were then pooled and are shown in figure 3.3.

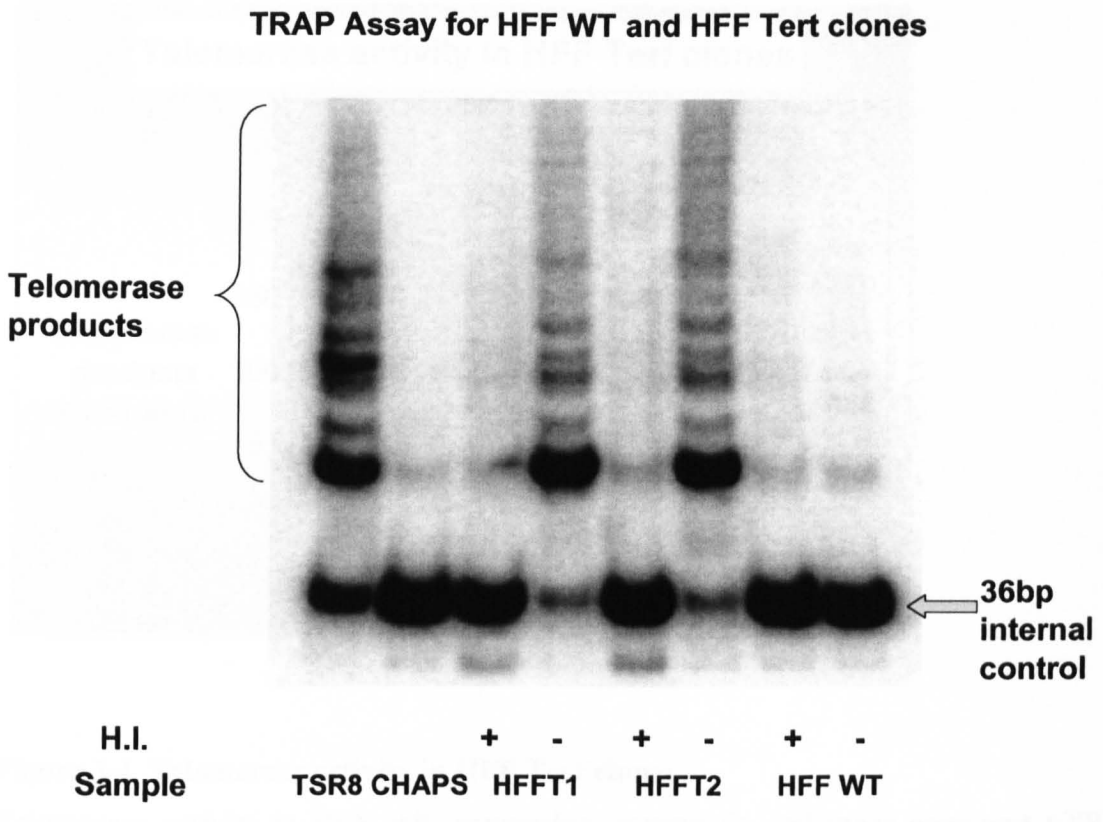


Figure 3-3. TRAP assay for HFF TERT clones and HFF WT cells

Telomere repeat amplification protocol assay for HFF TERT clones and HFF WT early pass cells. (H.I.; heat inactivation). Lanes are labelled and show positive control (TSR8), negative control (CHAPS lysis buffer) and heat inactivation control lanes as indicated. HFFT1 and HFFT2 protein extracts show telomerase products while HFF wild type produce none.

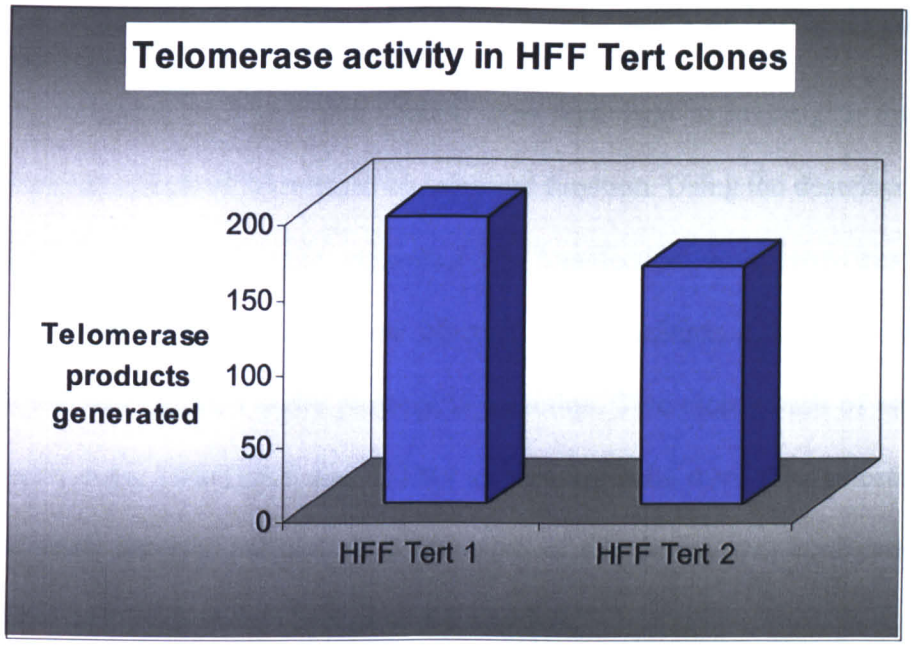


Figure 3-4. Telomerase activity in HFF Tert clones

Telomerase activity in HFF cells expressing puromycin resistance gene and hTERT gene. This is expressed as telomerase products generated as amplified in the TRAP assay PCR method as described in chapter 2. Wild type HFFs show no telomerase catalytic activity

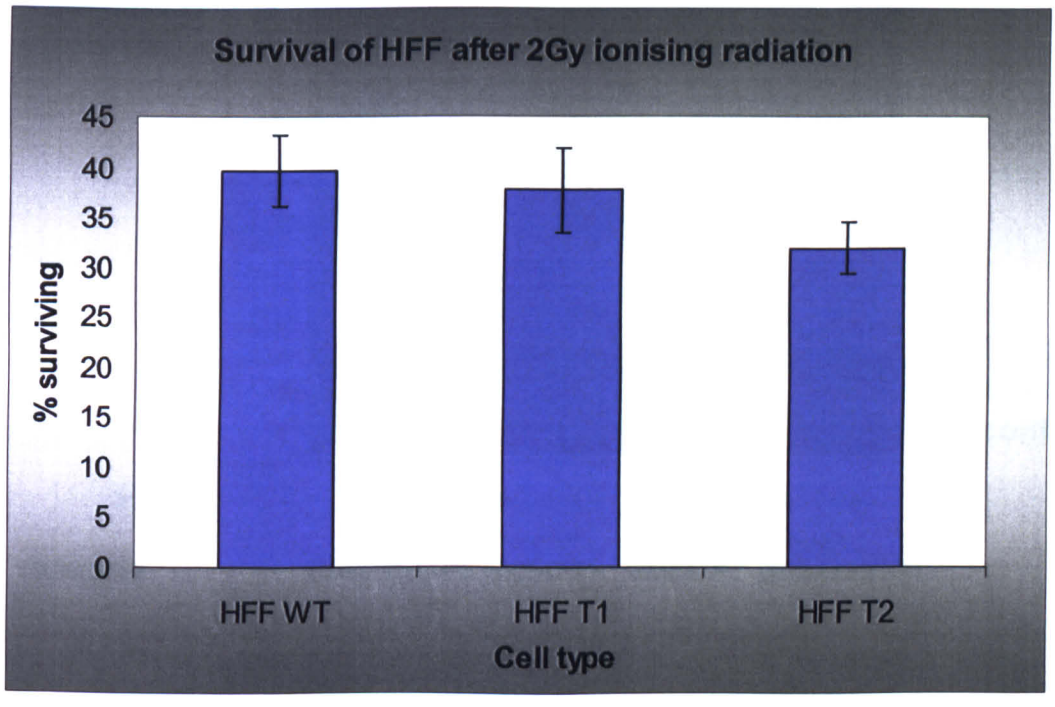


Figure 3-5. SF2 for HFF WT and HFF Tert 1 and HFF Tert 2

Survival fraction at 2 Gy for early pass wild type HFFs vs two pools of hTERT HFF clones. Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. There is no statistically significant difference for SF2 HFF1 and HFFT2 compared to wild type controls (Mann Whitney U test).

3.6. Radiosensitivity of GM847cells with ectopic expression of telomerase.

The immortal ALT cell line GM847 was then used to investigate the specificity of the observed effects of telomerase on telomere function. Using the described method, clones of GM847 fibroblasts were generated by transfection with retrovirus carrying hTERT, DNhTERT and drug resistance plasmid only. Multiple clones of each were then ring cloned and cultured under puromycin selection. Two clones each of antiobiotic resistance vector only, DNhTERT and hTERT expressing cells were then selected and cultured for radiation survival calculation. Expression of telomerase was confirmed using the TRAP assay and this is shown in figures 3-5 and 3-6.

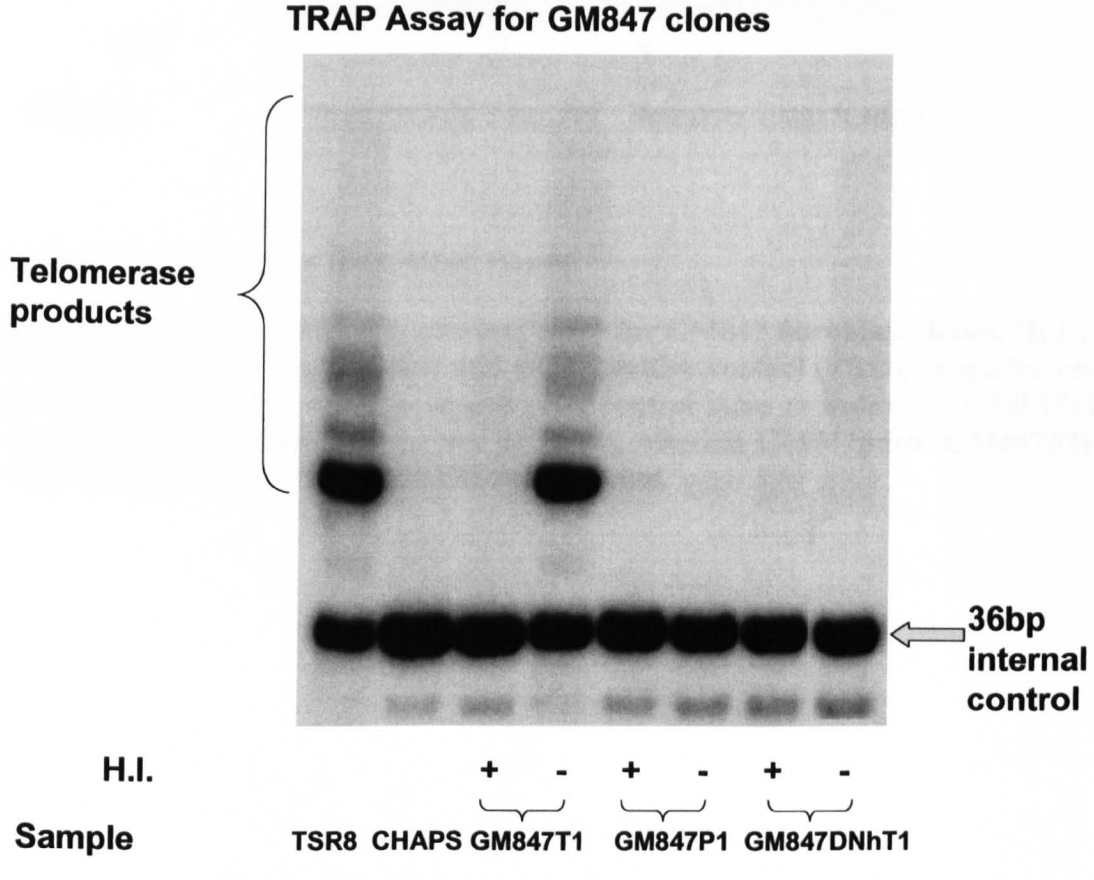


Figure 3-6. TRAP assay for GM847 clones.

Telomere repeat amplification protocol assay for GM847 fibroblast clones. (H.I.; heat inactivation). Lanes are labelled and show positive control (TSR8), negative control (CHAPS lysis buffer) and heat inactivation control lanes as indicated. GM847TERT clone (GM847T1) shows telomerase products, whereas GM847puro (GM847P1) and GM847DNhTERT (GM847DNhT1) clones do not.

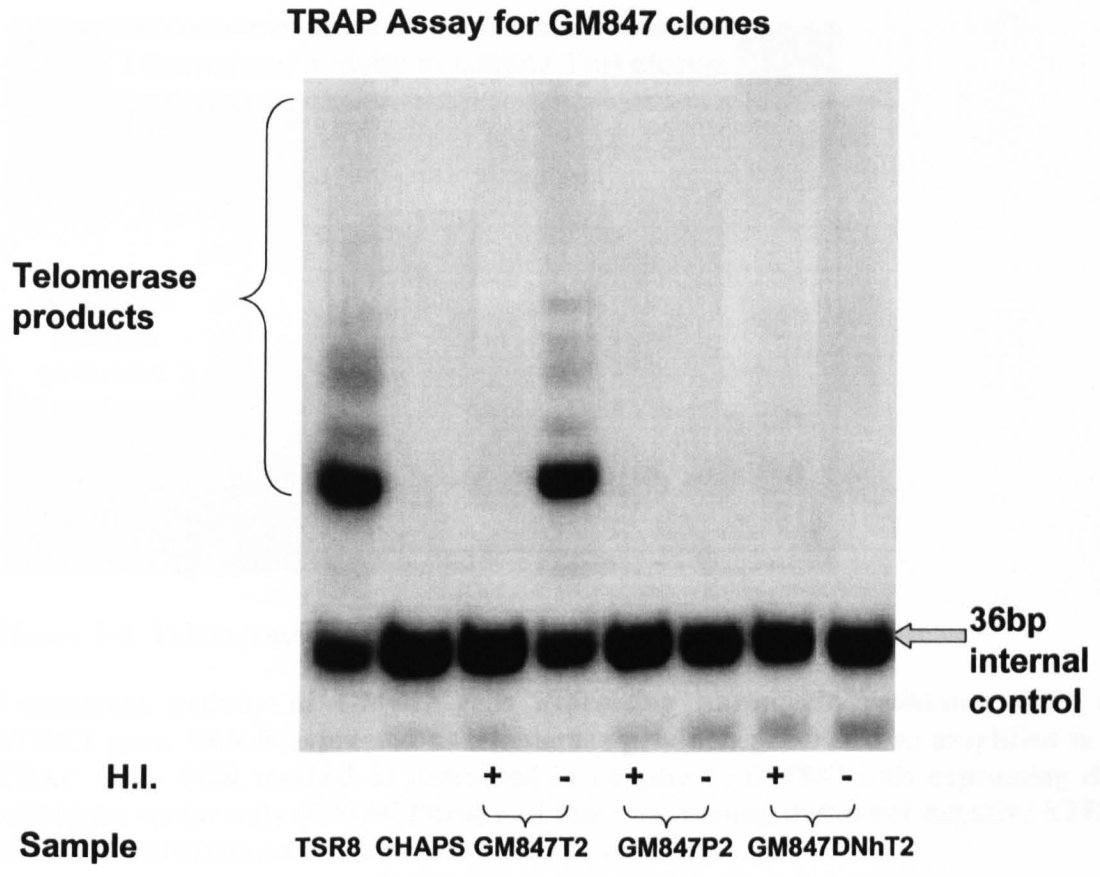


Figure 3-7. TRAP assay for GM847 clones

Telomere repeat amplification protocol assay for GM847 fibroblast clones. (H.I.; heat inactivation). Lanes are labelled and show positive control (TSR8), negative control (CHAPS lysis buffer) and heat inactivation control lanes as indicated. GM847TERT clone (GM847T2) shows telomerase products, whereas GM847puro (GM847P2) and GM847DNhTERT (GM847DNhT2) clones do not.

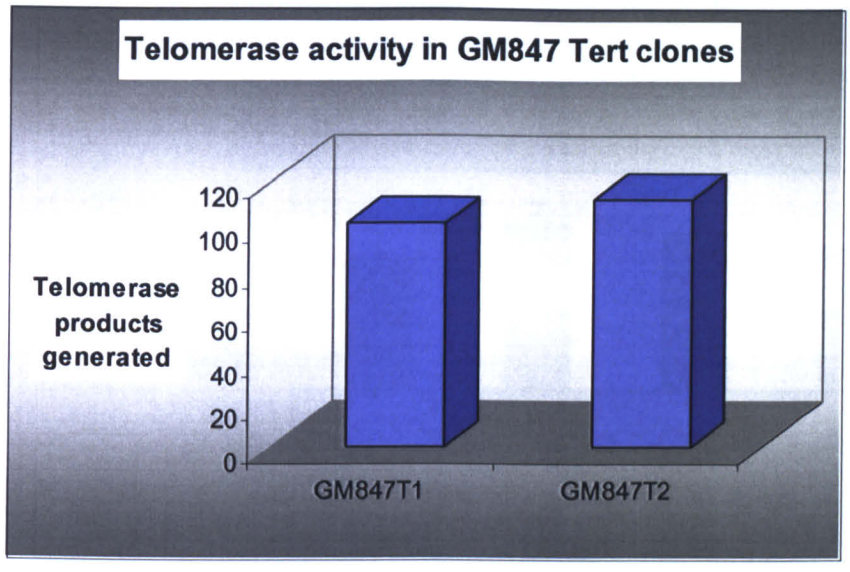


Figure 3-8. Telomerase activity in GM847 cells

Telomerase activity in GM847 cells expressing puromycin resistance gene and hTERT gene. This is expressed as telomerase products generated as amplified in the TRAP assay PCR method as described in chapter 2. GM847 cells expressing drug resistance vector only (GM847Puro) and those expressing dominant negative hTERT (GM847DNhT) show no telomerase catalytic activity.

Figure 3-7 shows the level of telomerase products generated in the ectopic telomerase expressing cells shown in the assays illustrated in figures 3-5 and 3-6. Survival fraction at 2Gy was then calculated and is illustrated in figure 3-8. No significant differences were observed in SF2 between clones expressing empty vector only, hTERT or DNhTERT.

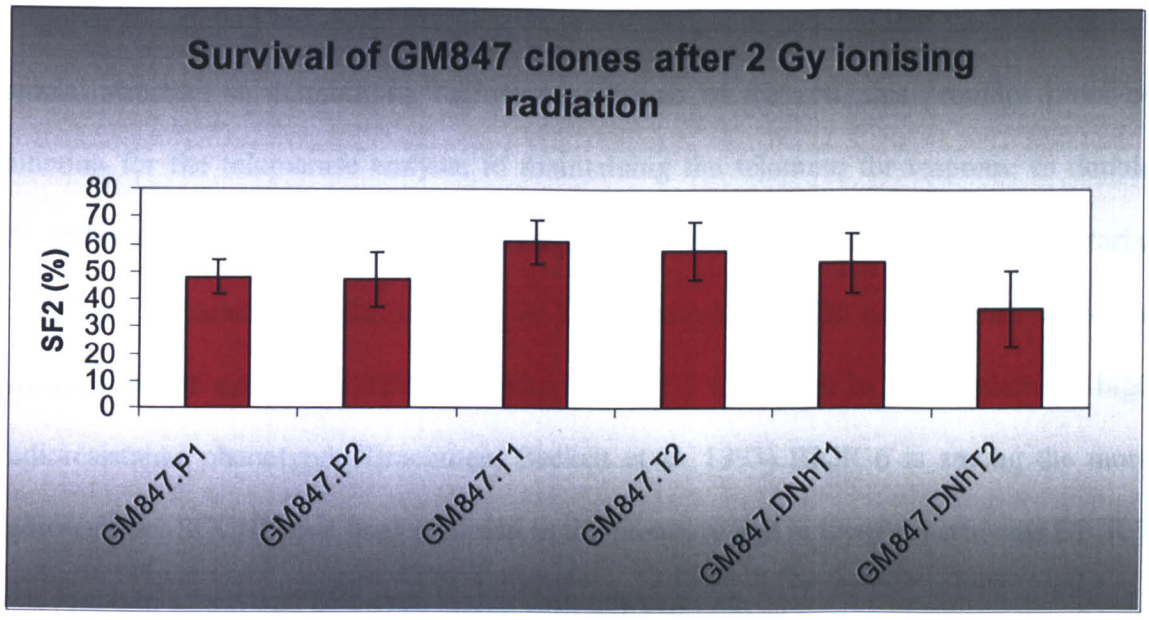


Figure 3-9. SF2 for GM847 fibroblast clones.

Survival fraction at 2 Gy for GM847 fibroblasts expressing antibiotic resistance gene only (GM847puro), hTERT (GM847T) and dominant negative hTERT (GM847DNhTERT). Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Differences in SF2 are not statistically significant for comparisons between any of the clones (P>0.05 in all comparisons; Mann Whitney U test).

3.7. Discussion:

This data shows a clear reduction in radiosensitivity of a SCCHN cancer cell line (BICR 7) when the endogenous level of telomerase is increased by ectopic expression of the hTERT gene product; the telomerase enzyme. This has been shown to restore telomere function in these cells as evidenced by increased TRF length, reduced anaphase bridge index and improved cloning efficiency. (Gordon, Ireland et al. 2003) Control cells (BICR 7Neo) were not rendered radioresistant by the stable transfection and clonal selection process. Using BICR 6 in this experiment demonstrates the absence of a radioprotective benefit with supraphysiological levels of telomerase in a cancer cell line which already expresses adequate levels of telomerase for telomere maintenance. This is the first evidence of the effect of manipulating telomerase in eukaryotic cancer cells with regard to the cellular

response to ionising radiation. Building on previous data suggesting that the telomere is a crucial structure in determining radioresponsiveness of the cell, this provides proof of function for the telomerase enzyme in maintaining the telomere for response to double strand break DNA damage. While it is likely that overall radiosensitivity is a multifactorial feature of mammalian cells, in these *in vivo* derived SCCHN cells a clear effect is demonstrated in enhancing radiosensitivity in BICR 7 to what is in fact a relatively high radioresistance phenotype. (Brachman, Beckett et al. 1993) BICR 6 is among the more radioresistant SCCHN cell lines recorded in the literature and in these experiments BICR 7 was found to achieve an SF2 even higher than this cell line.

In order to assess the specificity of this effect on cancer cells only, early pass HFFs were compared to HFFs stably transfected with the hTERT expression vector. Here in fact SF2 was shown to be reduced in the hTERT cells, although differences for the two clones used were not statistically different from wild type cells. Thus the telomerase protective effect is not seen in normal cells with long (10-15kb) telomeres and no *in vitro* evidence of telomere dysfunction.

The specificity of the effect on telomere dysfunction in telomerase dependant SCCHN cells was investigated by transfecting ALT cells (GM847) with hTERT. The effect of ectopic expression of hTERT in these cells has been recently reported. (Perrem et al) The ALT mechanism has been shown to be suppressed in telomerase expressing variants, although the telomerase enzyme *per se* was shown not to be the repressing factor. While GM847 cells have long and heterogeneous telomeres, expression of telomerase lengthens the shortest telomeres. Despite this effect these experiments using clones of GM847 cells expressing hTERT and the dominant negative hTERT genes showed no significant variation from those expressing the puromycin resistance vector only. Thus the radioprotective effect of telomerase is shown to be specific to cancer cells maintaining the shortened, post-senescent telomere with the telomerase enzyme complex and not to operate in ALT cells.

The key aim of this research is to investigate the translational value of manipulation of telomerase in producing a cell killing effect that is specific to cancer tissue. The next question to attempt to answer is the effect of inhibition of the telomerase enzyme in SCCHN cells on radiosensitivity. Should this provide an increase in cell death with ionising radiation then there may be a potential therapeutic benefit for SCCHN patients with a telomerase inhibition strategy.

Chapter 4.

Results.

Modulation of SCCHN radiosensitivity by telomerase inhibition.

4.1 Introduction

In this phase of investigation we aimed to assess the effect of telomerase inhibition on radiosensitivity of SCCHN. Our aim was to achieve inhibition of the catalytic activity of the telomerase enzyme complex, to assess the impact of that inhibition on telomere function and to then assay changes in radiation survival.

4.1.1 Telomerase inhibition with dominant negative hTERT.

A number of investigators have successfully achieved telomerase inhibition in experimental systems. Among the methods used, dominant negative mutants which inhibit endogenous telomerase function have been among the most effective. (Hahn, Stewart et al. 1999; Zhang, Mar et al. 1999) Several point mutants in the telomerase catalytic subunit hTERT which substantially reduce telomerase activity have been previously identified. (Harrington, Zhou et al. 1997; Nakamura, Morin et al. 1997; Weinrich, Pruzan et al. 1997) This information allowed the development of experimental systems where such mutants were shown to reliably inhibit telomerase. One group used a muristerone inducible system to produce expression of the mutant telomerase (Zhang, Mar et al. 1999) while in another, an amphotrophic retroviral vector transferred either DNhTERT or a drug resistance marker only into cells and produced stable expression of the mutant protein. (Hahn, Stewart et al. 1999) Both of these experimental systems have been shown to successfully inhibit telomerase, eliminating tumorigenicity and producing tumour cell death. The DNhTERT method of telomerase inhibition was selected for investigation with SCCHN in this study.

4.1.2. Dominant negative hTERT

The dominant negative hTERT used in these experiments contains two amino acid substitutions in the third reverse transcriptase motif. Residues 710 (aspartate) and 711 (valine) are replaced with alanine and isoleucine respectively. This catalytically inactive protein has been shown to successfully inhibit endogenous telomerase activity in cancer cells producing a reduction in telomere length and ultimately loss of tumour cell invasion in animal models and tumour cell death. (Hahn, Stewart et al. 1999)

4.1.3. DNhTERT in SCCHN

For this part of the investigation we infected several SCCHN cell lines with a retrovirus encoding either the empty pBabe puro vector, or the same vector containing pBabehTERT or pBabeDNhTERT. Infection was successful in BICR 6 and a number of clones were isolated as described in chapter 2. This cell line has robust growth characteristics and radiation survival at the high end of the range for SCCHN cell lines (SF2 = 71.4%, SF4 = 24.3%; see chapter 5). These cells express high levels of telomerase in comparison to other SCCHN and have correspondingly low levels of telomere dysfunction. (Gordon, Ireland et al. 2003) Initial experiments using the DNhTERT construct in the pBabepuro plasmid with the phoenix A retroviral vector system produced a number of clones of the BICR 6 line as described in detail in chapter 2. Early data obtained from cloning efficiency experiments did show some evidence of radiosensitisation of DNhTERT clones in comparison to controls (empty vector BICR6 Puro and high level telomerase expressing BICR6 Tert). Unfortunately however, after a number of population doublings, while puromycin resistance was retained by all 12 DNhTERT clones, telomerase enzyme activity was retained in the surviving cell populations in all cases, although to differing levels of catalytic activity. (Fig 4-1 – 4-3)

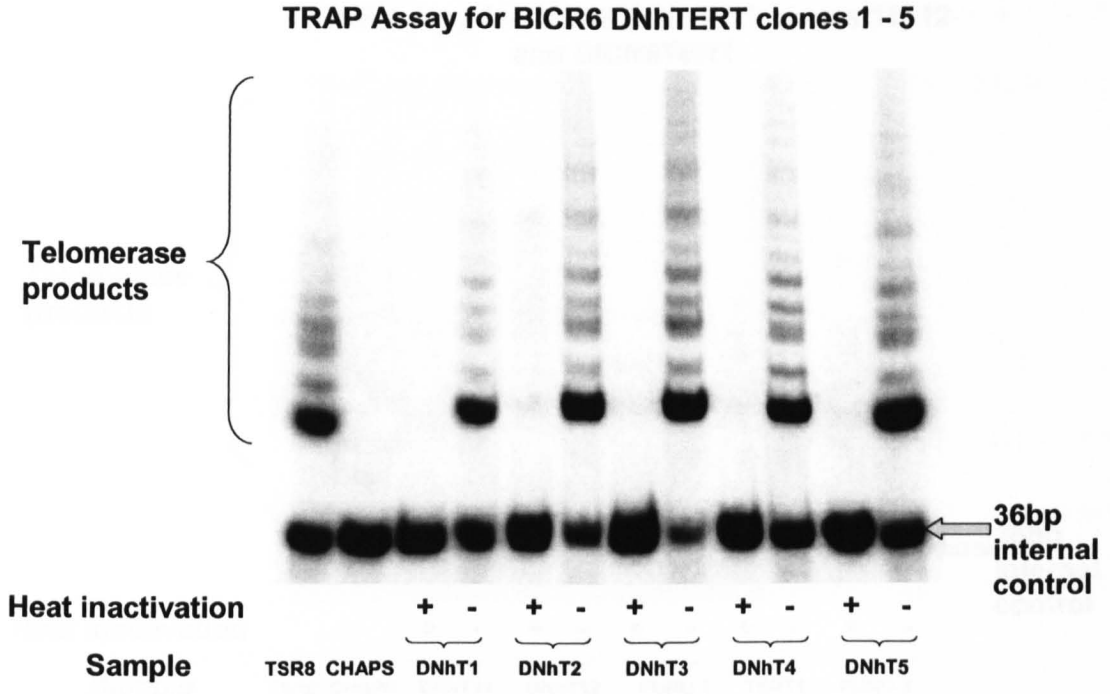


Figure 4-1. TRAP assay for BICR6 DNhTERT clones 1 - 5

Telomere repeat amplification protocol assay showing DNA products generated by telomerase activity in BICR 6 DNhTERT clones 1 - 5

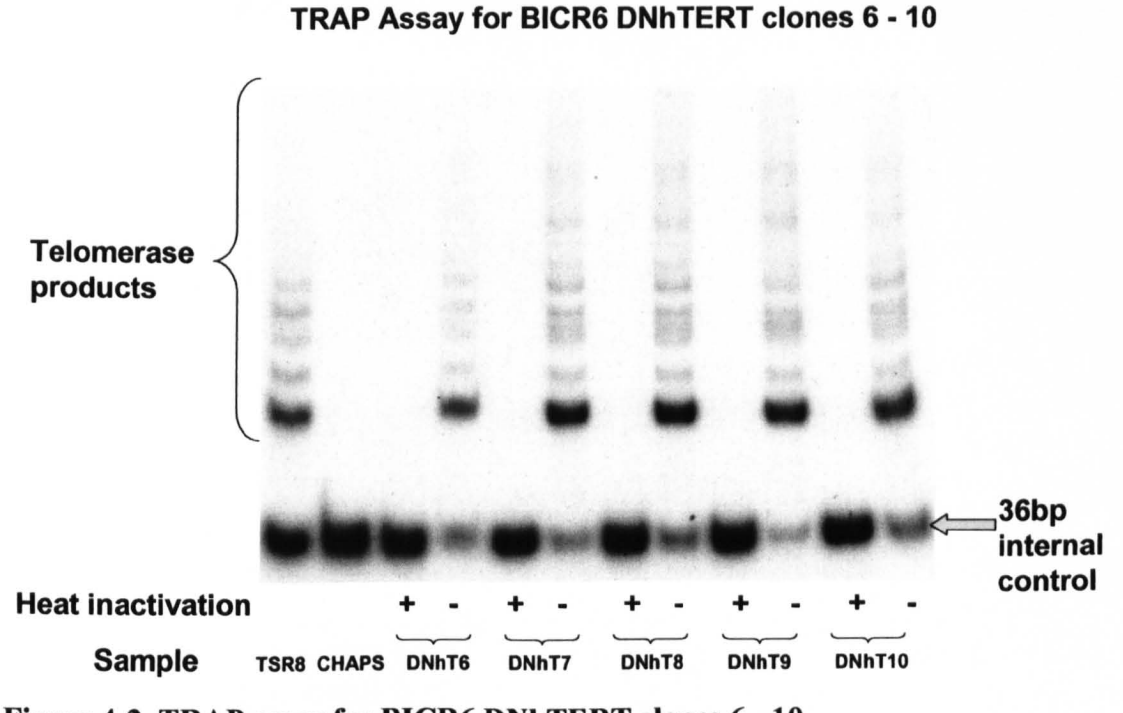


Figure 4-2. TRAP assay for BICR6 DNhTERT clones 6 - 10

Telomere repeat amplification protocol assay showing DNA products generated by telomerase activity in BICR 6 DNhTERT clones 6 – 10.

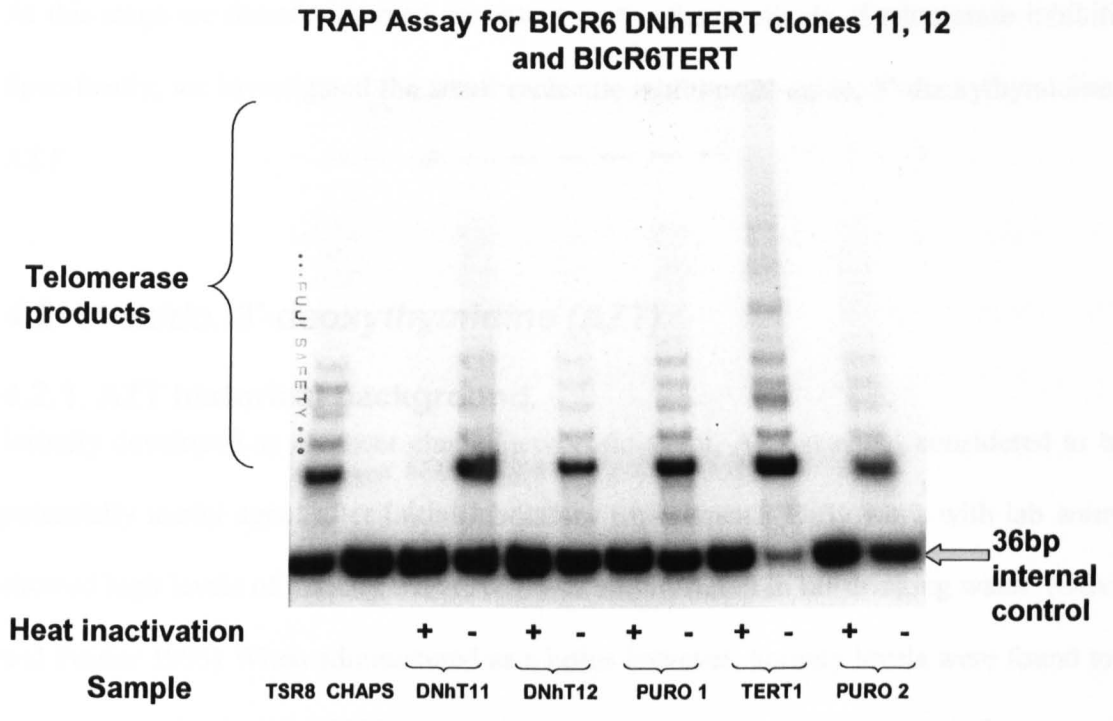


Figure 4-3. TRAP assay for BICR6 DNhTERT clones 11 and 12 and Puro 1, Puro 2 and Tert 1

Telomere repeat amplification protocol assay showing DNA products generated by telomerase activity in BICR 6 DNhTERT clones 11 and 12 and BICR 6 Puro 1 and 2 and BICR 6 TERT.

At this stage we therefore turned our attention to other methods of telomerase inhibition. Specifically, we investigated the small molecule inhibitor 3'-azido, 3'-deoxythymidine, or AZT.

4.2 3'-azido, 3'-deoxythymidine (AZT)

4.2.1. AZT historical background.

Initially developed as a cancer chemotherapeutic agent, AZT was not considered to be a potentially useful agent after initial laboratory experiments. Early work with lab animals showed high levels of toxicity when AZT was administered in the drinking water. (Olivero and Poirier 1993) When administered as a bolus however, toxicity levels were found to be much lower. In the mid 1980s researchers tackling the human immune deficiency virus associated AIDS epidemic found that AZT inhibited viral reverse transcriptase. A subsequent phase II trial on the effectiveness of AZT in HIV associated disease was stopped early because of the effectiveness of the drug and the drug entered clinical practice in 1987.

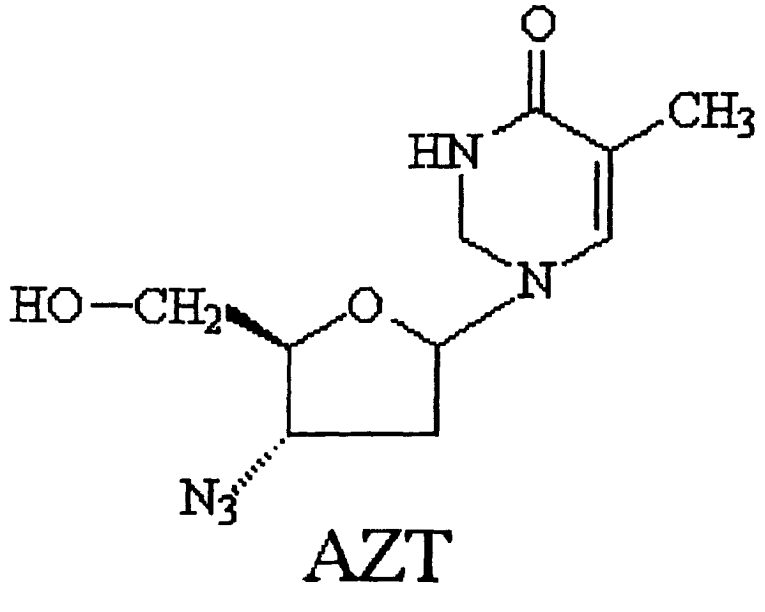


Figure 4-4. Chemical structure of AZT.

The chemical structure of AZT is shown in figure 4.1. The drug represents a false nucleotide which resembles thymidine; the 3'-hydroxy group being replaced with an azido (N_3) group. On entry to the cell it is phosphorylated by thymidine kinase to AZT 5'-triphosphate which is the active metabolite of the drug. This is incorporated into growing DNA strands instead of thymidine triphosphate and causes premature chain termination. The drug is known to have a low affinity for DNA polymerases α, β, and γ and also mitochondrial polymerase γ but a high affinity for HIV-1 reverse transcriptase and therefore has a high degree of specificity of action. (Melana, Holland et al. 1998)

4.2.2 AZT in current clinical practice.

When first introduced into clinical practice in 1987 it was thought that the optimum dosage regimen would be 100mg every four hours with no overnight break. This was modified in light of the side effect profile of the drug in clinical practice. Currently AZT is utilised to avert foetal maternal transmission of HIV infection and as one component of combination therapy to treat HIV related disease.

4.2.3 AZT in cancer

The early development of AZT as a cancer therapeutic agent was followed by some work in gastrointestinal cancers and some tumour regression in colorectal cancers has been reported. (Posner, Darnowski et al. 1990; Posner, Darnowski et al. 1992; Beitz, Damowski et al. 1995; Falcone, Lencioni et al. 1997) In these studies, the effect of AZT on telomerase was not investigated.

The catalytic component of the telomerase enzyme complex has been shown to be closely related to other reverse transcriptases. (Lingner, Hughes et al. 1997) (Nakayama, Tahara et al. 1998) During the course of the investigation of the role of telomerase in cancer cells, numerous known reverse transcriptase inhibitors have been studied. Among these, AZT has been used to successfully inhibit telomerase in B-cell and T-cell lines (Strahl and Blackburn 1996) (Melana, Holland et al. 1998), breast cancer cell lines (Melana, Holland

et al. 1998) and uterine endometrial cancer lines (Murakami, Nagai et al. 1999). Further studies have examined the effect of AZT inhibiting telomerase with other telomere interacting agents (Rha, Izbicka et al. 2000) (Mo, Gan et al. 2003).

We investigated the effect of AZT on telomerase catalytic activity in BICR 6 SCCHN cells. As controls, cells transfected with hTERT and stably expressing supraphysiological levels of the telomerase enzyme were used. We then assayed the effect of this inhibition on telomere function by assessment of anaphase bridge scoring. Finally, radiation survival after 2 Grays of ionising radiation was assessed for cells treated with AZT.

4.3. Telomerase inhibition in SCCHN with AZT

4.3.1. TRAP assay data for BICR6 wild type and BICR6Tert with AZT.

Appropriate AZT dosages for these experiments were obtained by culturing cells in the drug at a variety of dose levels. Doses selected were the highest which still allowed cells to reach confluence in twelve-well plates, as described in detail in chapter 2. The levels of telomerase inhibition were established by TRAP assay of protein extract from cultured cells. The Telomere repeat amplification protocol used for these experiments (TRAPese, Intergen Co.) is a PCR based, semiquantitative assay of telomerase activity in whole protein extract from cells. In order to establish the linear range for the BICR cell lines, preliminary work was carried out on prepared cellular extracts within the R9 research group by Dr E K Gordon. These experiments showed linearity with protein inputs of between 0.2-2 μg. (Gordon, Ireland et al. 2003)

The protein input for the BICR6 cell line used in the telomerase inhibition experiments was at the lower end of the pilot range (0.2-2μg). Pilot experimental data showed that inputs of 0.2μg for BICR6 and BICR6Tert produced telomerase products inside the stated linear range for the TRAP assay (1 – 300TPGs). These data are shown in figures 4.5 to 4.7. Clear evidence of inhibition of telomerase is shown in BICR 6 cells cultured for seven days in 50μM and 100μM AZT.

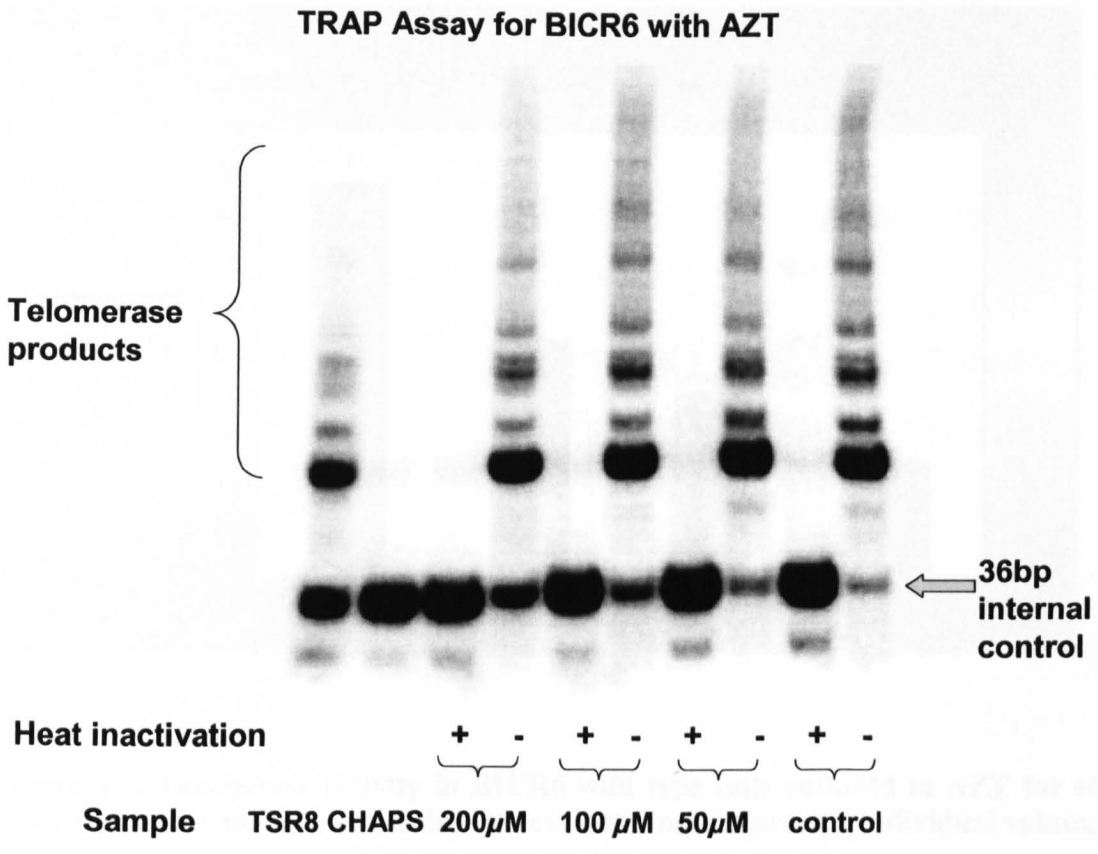


Figure 4-5. TRAP assay of BICR 6 cells cultured in AZT

DNA products generated by telomerase activity in protein extract from BICR 6 cell cultured in AZT for seven days. The intensity of the control 36bp band can be seen to increase with higher doses of AZT, reflecting reduced telomerase catalytic activity

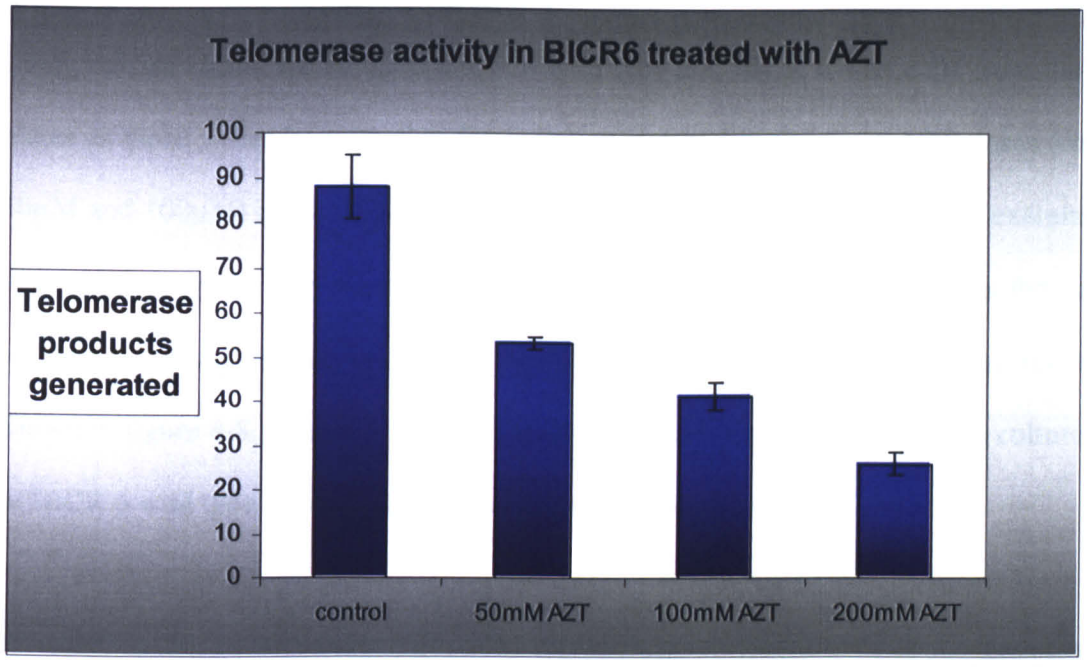


Figure 4-6. Telomerase activity in BICR6 wild type cells cultured in AZT for seven days. Values are the average of duplicates. Error bars represent individual values.

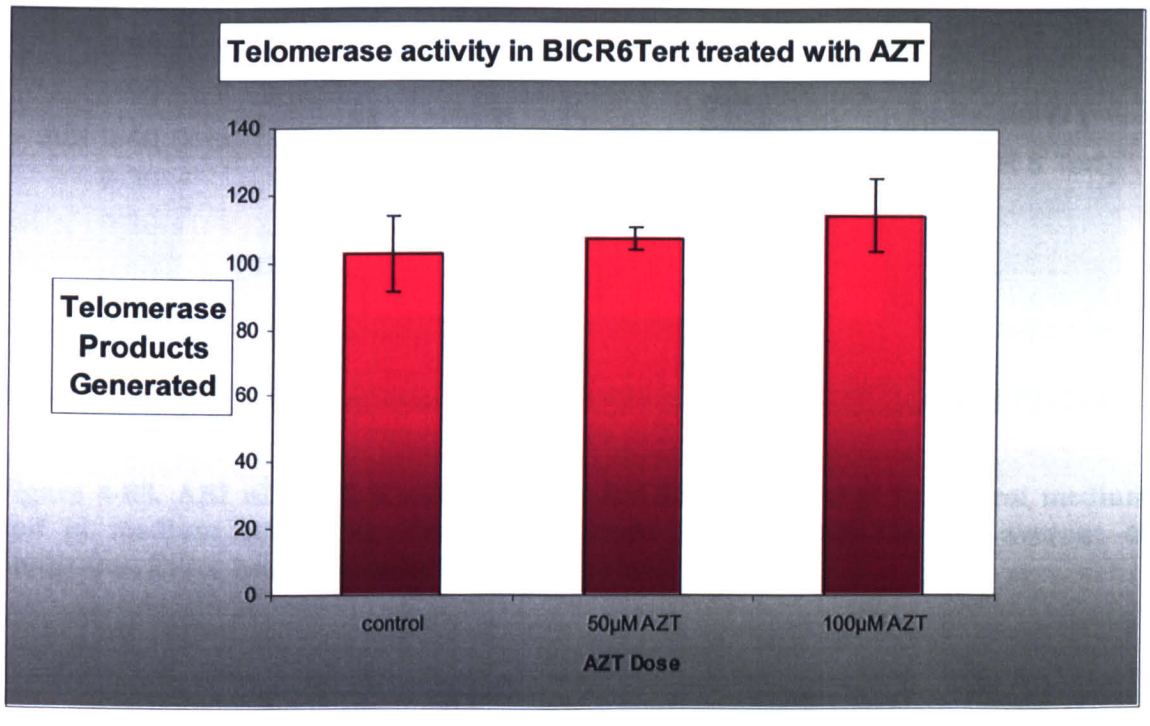


Figure 4-7. Telomerase activity in BICR6Tert cultured in AZT for seven days. Values are the average of duplicates. Error bars represent individual values.

4.3.2. Telomere function in BICR 6 cells cultured in AZT.

Using culture slides, we cultured BICR 6 wild type and BICR 6 Tert cells from the same plates as those under investigation for radiation survival in the three AZT doses (control, 50μM and 100μM) in identical conditions. These were fixed and stained and examined by light microscopy by two observers (the author and Dr E K Parkinson). Thus the ratio of bridged anaphase mitoses to normal anaphase mitoses was calculated. These results are shown in figure 4-8. Figure 4-9 shows three bridged anaphase mitoses from a culture slide of BICR 6 wild type growing in medium containing 50μM AZT.

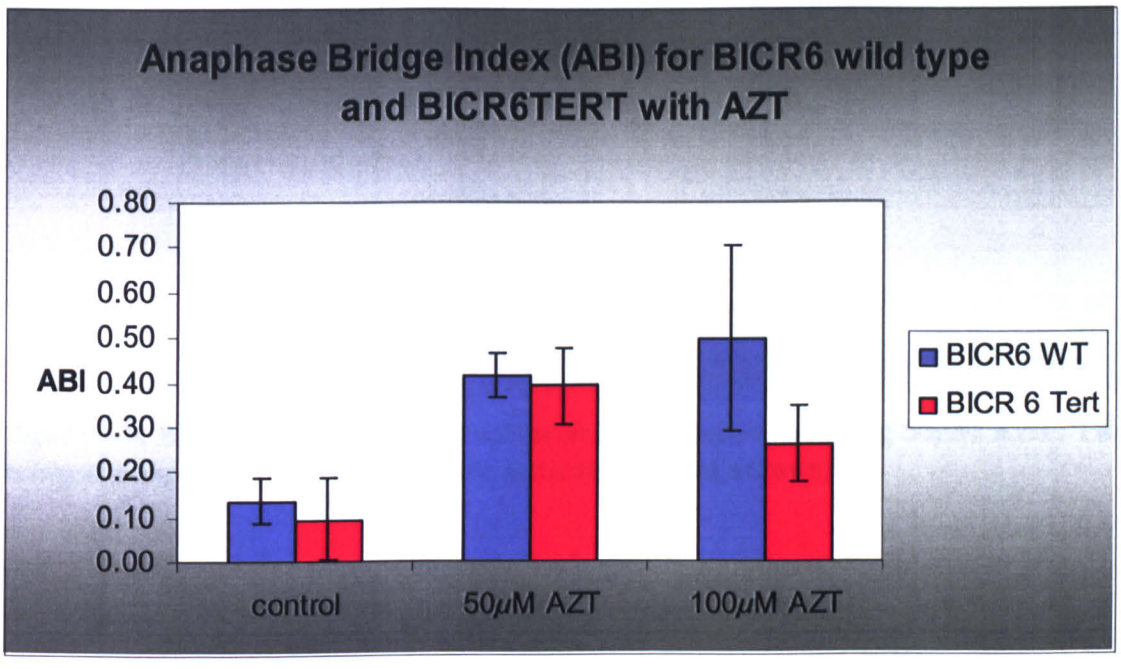


Figure 4-08. ABI of BICR 6 wild type and BICR6Tert cultured in control medium and in medium containing 50μM and 100μM AZT. Values are the average of duplicates. Error bars represent individual values.

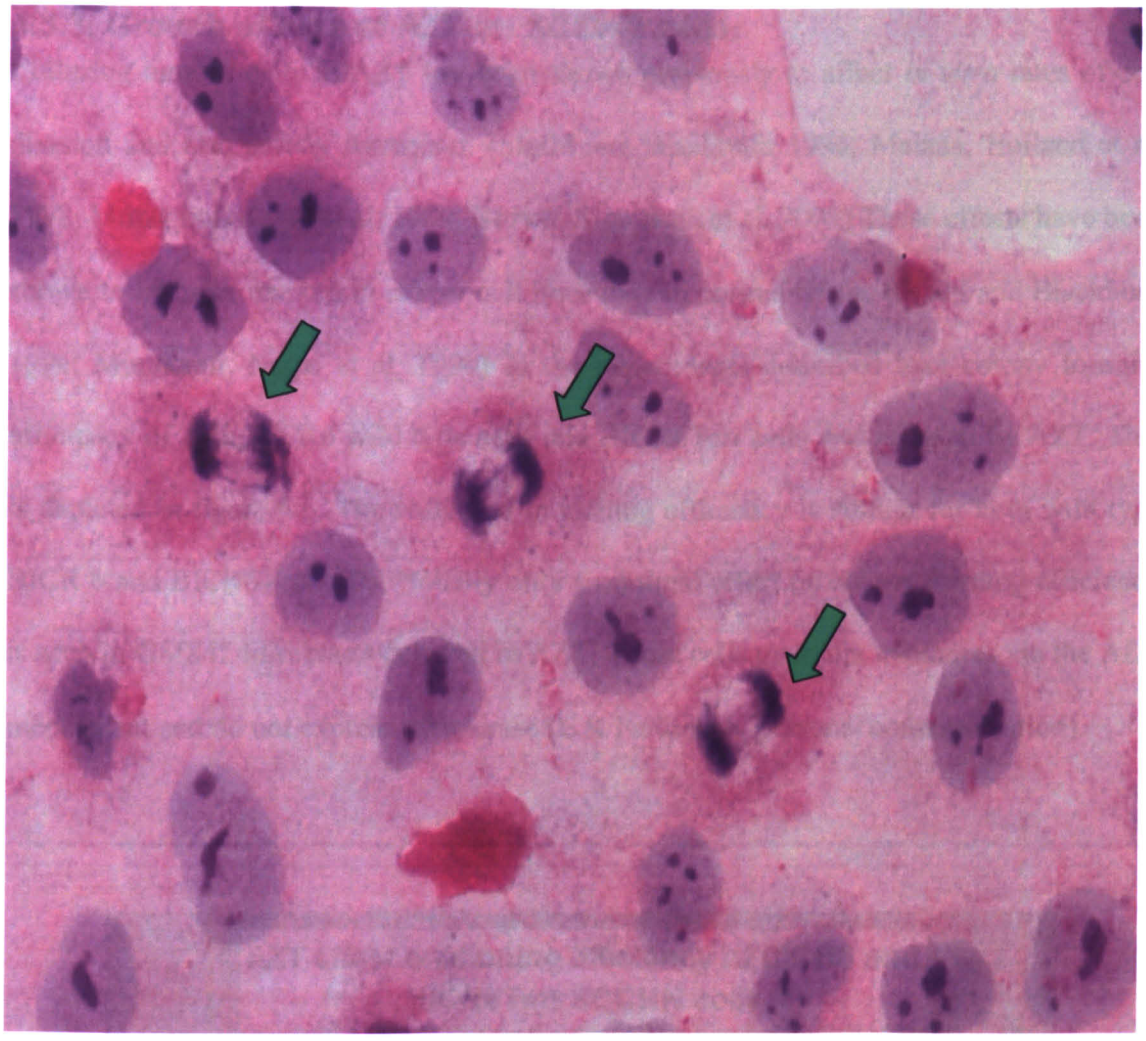


Figure 4-9. BICR 6 wild type cells cultured in medium containing 50μM AZT. Three bridged anaphase mitotic figures are indicated (green arrows)

While telomere dysfunction appeared to increase with AZT mediated telomerase inhibition in BICR 6 wild type cells, there was also an apparent increase in telomere dysfunction in control BICR6Tert cells.

4.3.3. Growth effects of AZT on SCCHN cells.

Culturing tumour cells in AZT has been shown previously to affect *in vitro* rates of cell division and also colony formation. (Strahl and Blackburn 1996; Melana, Holland et al. 1998; Murakami, Nagai et al. 1999; Brown, Sigurdson et al. 2003) These effects have been shown to be more marked in cancer cell lines than in normal tissue. (Strahl and Blackburn 1996; Murakami, Nagai et al. 1999) In the experiments described here colony forming efficiency of both BICR6 wild type cells and BICR6Tert cells was affected by AZT. This is depicted in figure 4.6. The reduction in cloning efficiency is similar for both wild type BICR 6 and BICR6Tert cells. Notably only a minimal effect on colony forming efficiency is seen in GM847 fibroblasts cultured in AZT. These cells maintain telomeres via the ALT mechanism and do not express telomerase (E K Parkinson, personal communication).

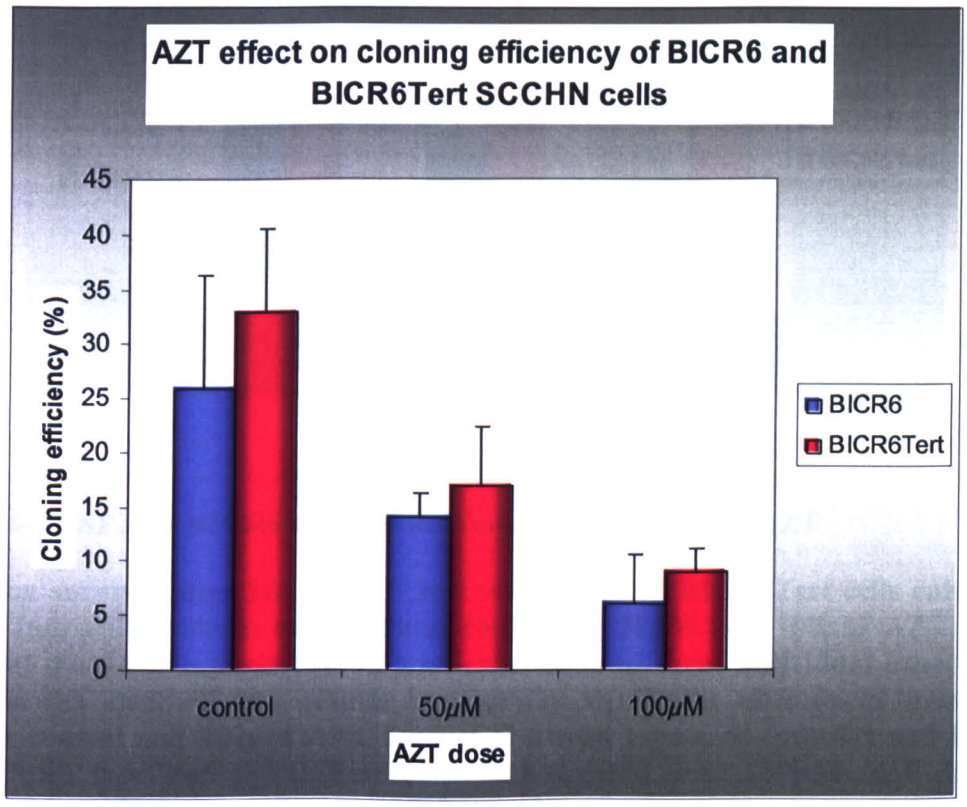


Figure 4-10. The effect of AZT on colony forming efficiency in BICR6 and BICR6Tert. Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results Different values for each dose and each cell line are not significant statistically ($p>0.05$ in all cases).

4.3.4. Radiosensitivity of BICR 6 cells cultured in AZT.

Having established that telomerase catalytic activity was indeed reduced in a dose dependent fashion by AZT and that this was affecting telomere function, we then investigated any change in radiation survival in these cells. This data is depicted in figure 4-11. which combines SF2 data for BICR6 wild type and BICR6TERT cells for control cells and for each of the AZT doses examined. All experiments were repeated in triplicate, with a total of six replicates for each value.

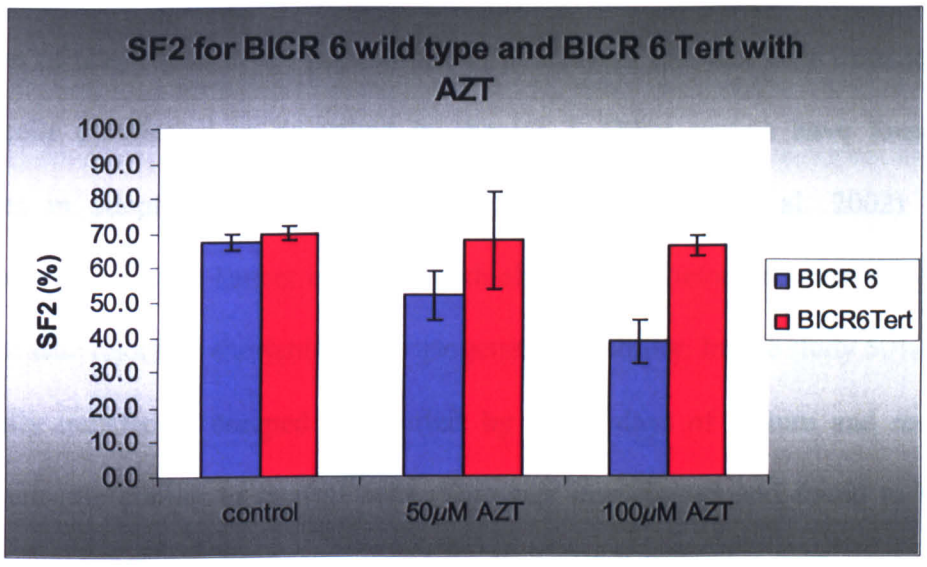


Figure 4-11. SF2 for BICR6 and BICR6 Tert cells treated with AZT

Radiation survival after 2Gy for BICR 6 wild type and BICR6Tert cells cultured in control (normal) medium and medium containing 50μM and 100μM AZT. Values represent means of experiments in triplicate. Each of three individual experimental values is the mean of two results Statistically significant differences were found between control and 100μM AZT for BICR 6 wild type cells ($p<0.01$) and between BICR6 wild type and BICR6Tert cells both treated with 100μM AZT ($p<0.01$); Mann-Whitney U Test.

This bar chart shows the clear reduction in SF2 for wild type BICR6 cells with AZT. This reaches statistical significance in these experiments at 100μM AZT ($p<0.01$). Note that

surviving cells are expressed as a percentage compared to non-irradiated controls. Actual cell survival with combined AZT and irradiation is much less in BICR 6 (mean 8.6% survival in 100μM AZT at 2 Gy). In experiments carried out using the same method with GM847 cells no change in radiosensitivity at 2Gy was seen with AZT in a range of concentrations up to 100μM (E K Parkinson, personal communication).

4.4. Discussion.

Initial work with DNhTERT mutant expressing cells proved unsuccessful due to continued expression of telomerase in the populations of puromycin resistant cells obtained by ring cloning cells transfected as described in chapter 2. Other groups have found similar difficulties in adopting this strategy. (Delhommeau, Thierry et al. 2002) Reducing telomerase expression in cancer cell lines with already short telomeres has been shown to result in drastic telomere shortening and cytogenetic instability. In one study 50% of clones successfully transfected escaped from crisis by 15-35 days of culture and recovered a proliferation rate similar to control cells. This was investigated and found to be due to telomerase reactivation, mainly by loss of the DNhTERT transcript, with maintenance of other components of the transfected plasmid, but also by transcriptional upregulation of endogenous hTERT. (Delhommeau, Thierry et al. 2002) Such findings emphasise the level of selective pressure produced by the crisis phenotype and the facility with which some cancer cells can upregulate telomerase. This is of course a concern regarding the use of telomerase inhibition therapeutically and a further driver toward combination therapies which will reduce the opportunity for emergence of resistant clones of cells. (Hanahan and Weinberg 2000)

In this series of experiments, we chose not to investigate in detail the means by which telomerase expression had been re-established or maintained, but rather to press forward

inhibiting telomerase by other means in order to address the question of reduced telomerase expression and the effect on telomere function and radiation survival in SCCHN.

The growth inhibitory effect of AZT on cancer cells has been described by a number of authors in a variety of different cancer cells lines. (Strahl and Blackburn 1996; Melana, Holland et al. 1998; Murakami, Nagai et al. 1999; Rha, Izbicka et al. 2000; Gan, Mo et al. 2002; Brown, Sigurdson et al. 2003) Notably, this effect on cell replication has been described as much less marked in normal cells. (Melana, Holland et al. 1998) In our experiments, AZT at 50μM and 100μM concentrations caused significant reduction in colony forming efficiency in wild type SCCHN tumour cells. A lesser but similar effect was observed in cells expressing ectopic hTERT. While some of this effect may be due to non-specific toxicity, there is only a small effect on colony forming efficiency in ALT cells at the highest AZT dose used here (Dr E K Parkinson, personal communication). Thus ectopic hTERT is not able to significantly block the growth inhibitory effects of AZT over the short time frame of exposure in these experiments. Interestingly, in longer term experiments examining the effect of AZT on colon cancer cells, the most marked effect is seen early and reduces with time. (Brown, Sigurdson et al. 2003)

Telomerase catalytic activity, as assessed by the PCR based TRAP assay was shown to be reduced in the presence of the small molecule inhibitor AZT. These experiments have therefore shown that telomerase inhibition is achievable using this reverse transcriptase inhibitor in BICR6, an *in vivo* derived SCCHN cell line. The data obtained here are comparable to the levels of telomerase inhibition seen in work carried out on other lines with AZT. (Brown, Sigurdson et al. 2003)

Telomere function appeared to reduce with telomerase inhibition due to AZT contained in culture medium. This occurred in a dose dependent fashion with wild type BICR6 cells and in a short time frame. BICR 6 are known to have telomeres of between 4-6kbp (Gordon, Ireland et al. 2003) and so reduction of telomere length alone (losing 150-250 base pairs

per cell division) would not be predicted to have occurred sufficiently in such a short interval (seven days) to produce the level of telomere dysfunction observed. There was also a reduction in telomere function in control BICR6Tert cells. These cells have very high levels of telomerase expression and there was no detectable change in telomerase activity by TRAP assay when they were exposed to AZT. This is an interesting, if unpredictable finding, which perhaps reflects the complex nature of telomere capping. The data obtained are however not adequate to draw any firm conclusions regarding AZT induced changes in telomere function. AZT inhibits reverse transcriptase function by competing for the catalytic site of the enzyme and is relatively specific for enzymes of this type. (Melana, Holland et al. 1998) Again, where telomerase is inhibited effectively, a reduction in capping may reflect the further role(s) of the telomerase enzyme complex in maintaining cytogenetic stability in the context of the shortened telomeres of the cancer cell. There is however a further function of AZT in its incorporation into growing DNA chains, causing chain termination. It is possible that this function is contributing to the observed increase in ABI. A further consideration here is whether the incorporation of AZT into telomeric sequences could produce mutant telomeres and therefore account for change in ABI and reduction in cloning efficiency. Recent work has shown that the formation of such mutant telomeres in tumour cells inhibits tumour cell proliferation. (Kim, Rivera et al. 2001) Some more recent evidence has provided a mechanism for this and suggests that mutated telomeres in fact cause impaired chromosome separation and lead to a unique checkpoint response. (Lin, Smith et al. 2004) Here mutation of the RNA template of the telomerase enzyme complex caused incorporation of mutant telomeric DNA sequences in yeast. Although both growth rates and telomere profiles were variable, chromosome separation was consistently abnormal. These mutants displayed aberrant separation of sister chromatids at the centromere as well as the telomere, implying cell cycle checkpoint activation. Taking this evidence into account, it may well be the case that incorporation of AZT into the telomeres of telomerase proficient cells produces mutant,

dysfunctional telomeres. Thus we would anticipate that both wild type and ectopic hTERT expressing cells would be affected by mutated telomere DNA. This may therefore account, at least in part for the observed increase in ABI in BICR6Tert cells seen at 50μM AZT concentration.

Most interestingly in this part of our work, a clear increase in radiation induced cell death was observed when telomerase was inhibited in BICR6 wild type cells. This effect was not observed in BICR6Tert cells, where no telomerase inhibition could be detected. This finding is consistent with the intact functioning telomere having a critical role in radioresistance and complements the reverse finding of enhanced radioresistance in BICR7 with increased expression of telomerase complements in chapter 3.

The means by which this radiosensitisation is occurring is not entirely clear. Previous corroborative evidence in cell lines showed radiosensitivity increasing with telomere shortening. (McIlrath, Bouffler et al. 2001) Further, in the telomerase null mouse a radiosensitivity syndrome only developed after four to six generations, when telomeres had sufficiently shortened. (Goytisolo, Samper et al. 2000) Further work has shown that the shortened telomeres in these animals associate with double strand breaks induced by ionising radiation, interfering with correct rejoining of the broken ends. (Latre, Tusell et al. 2003) There had not been sufficient time for critical telomere shortening caused by cell division and the end replication problem in the experiments described in this chapter. Telomere function had certainly reduced in the AZT treated cells and reduced progressively with increasing AZT dosage. It is possible then that it is the intact, functioning telomere that is essential for radiation resistance. Telomere dysfunction can clearly be induced by shortening, but perhaps also by disruption of the mechanisms of maintenance of the short, post-senescence length telomere in the cancer cell. This maintenance may well involve the telomerase enzyme complex by means other than lengthening as has been suggested by other investigators. (Blasco 2002)

The exact mechanism by which telomere dysfunction induces radiosensitisation is not clear. Speculatively, the binding of DSB repair proteins (Mre11/Rad50/NBS1 and the DNAPK complex) may be hampered by the loss of telomere function. The T-loop, D-loop structure provides a means for the localisation of the 3' single-stranded DNA overhang. This may also be disrupted in the context of telomerase deficient telomere dysfunction in cancer cells and be implicated in radiosensitivity. The uncapped, post-senescent telomere may also be capable of combining with double strand break sites, as described by Latre *et al.* (Latre, Tusell et al. 2003) It is also possible that telomerase has a role to play in DSB repair occurring elsewhere in the genome and that telomerase inhibition impairs this.

Interestingly, while in these experiments we are examining the effect of ionising radiation in combination with telomerase inhibitor therapy, other workers have assessed the effect of other chemotherapeutic agents in the context of AZT telomerase inhibition. (Mo, Gan et al. 2003) Targeting telomeres with paclitaxel, in combination with AZT, confirms that in combination this approach is effective in specifically inducing cell death in telomerase positive cancer cells. Telomerase negative osteosarcoma cells were unaffected by AZT. The clearest implication of ionising radiation work in the context of chemotherapy would be for the use of double strand break inducing agents. These include doxorubicin, daunorubicin and actinomycin D.

We selected BICR 6 for AZT experiments as a typical SCCHN line derived as part of a series of cell lines at the Beatson Institute for Cancer Research. In terms of p53 and p16 status (both mutated) and upregulation of telomerase (expression toward the high end of the range found, with low levels of telomere dysfunction (Gordon, Ireland et al. 2003)) BICR 6 is a representative and relatively robust SCCHN cell line for experimentation. BICR 6 is also one of the most radioresistant lines we have studied. While proof of concept is provided by the experiments detailed in this chapter, testing the effect of AZT on telomerase expression across a wider panel of cell lines would give useful further data.

Animal experiments would also provide desirable further information. Implanting tumour cells in nude mice and assessing the effect of AZT in combination with ionising radiation in this context would help to further elaborate the usefulness of this approach.

In clinical practice, AZT is used to avert vertical transmission of HIV infection and also as a component of combivir® with other antiretroviral agents. Doses used in practice are between 500mg – 1500mg and are described as equivalent to 20 – 60μM. While previous phase II experiments using AZT in combination with other chemotherapeutic agents (5-FU) have been terminated due to toxicity, the targeted nature of ionising radiation therapy makes this a more attractive modality to combine with AZT mediated telomerase inhibition.

Chapter 5.

Results.

P53 codon 72 polymorphism and radiation survival in SCCHN.

5.1 Introduction

In this chapter experiments are described which examine two further parameters in relation to radiation survival in SCCHN. These are; codon 72 polymorphism and primary versus recurrent tumour of origin. Additionally the influence of observed telomere function in SCCHN cell lines. The first two of these recognise the complex nature of the cellular response to ionising radiation which may be affected by multiple factors while the third returns once again to telomere function across a panel of *in vivo* derived SCCHN lines

5.1.1. p53 codon 72 polymorphism in SCCHN.

Epidemiological studies have shown a statistically significant linear correlation between the frequency of the p53 Pro72 allele and latitude in several populations. (Beckman, Birgander et al. 1994; Sjalander, Birgander et al. 1995) Epidemiological data from Scotland does verify a higher incidence than the rest of the UK which cannot be entirely accounted for by the higher rates of tobacco use and alcohol consumption. (Scottish Cancer Registry 2004) This therefore implies some other genetic or environmental predisposing feature, the nature of which remains obscure at this time. Interestingly, data relating to the p53 codon 72 status in SCCHN occurring in the Scottish population is currently lacking.

5.1.2. p53 Arginine codon 72 is more effective in producing apoptotic cell death.

Recent in vitro studies have demonstrated the increased capability or the arginine polymorphic variant of p53 to induce apoptotic cell death. (Dumont, Leu et al. 2003) Codon 72 in fact lies in the proline rich domain which has been demonstrated to be essential for p53 to fully induce apoptosis. At least part of this increased apoptotic effect of the arginine variant is its superior localisation to the mitochondria accompanied by release of cytochrome C into the cytosol. This has been proposed to account, at least in part, for the reduced Arg72 incidence in regions with greater UV in the environment.

5.1.3. Mutated p53 Arg72 can bind to and inactivate both wild type p53 and p63 and p73 proteins.

Other p53 family members have been shown, at least when over expressed to bind to canonical p53 binding sites in vitro (Marin, Jost et al. 2000). These can also transcriptionally activate p53 target genes *in vivo*, therefore exerting the p53 associated tumour suppressive effects. (Kaelin 1999) Previous experimental work has demonstrated an increase in carcinogenicity when cells null for p53 are transfected with mutated p53. (Halevy, Michalovitz et al. 1990; Dittmer, Pati et al. 1993)Accumulation of mutated p53 in cancer cells can function in a dominant negative role by forming heterooligomers with wild-type p53 expressed by the second allele. Evidence presented recently shows that mutated p53 Arg 72 can both physically interact with and form complexes with p73 under physiological conditions and hence can inactivate it. (Marin, Jost et al. 2000) The p73 protein itself is only rarely mutated in cancer. Further, the cellular response to chemotherapeutic agents has been shown to be significantly affected by p53 codon 72 polymorphism. (Bergamaschi, Gasco et al. 2003)Cancers expressing the mutated Arg 72 variant have been demonstrated to have lower response rates to cisplatin than those expressing the mutated pro 72 variant.

5.1.4. p53 in the cellular response to DNA double strand breaks

The cellular reponse to stress induced by ionising radiation damage is mediated via DSBs which interact with kinases (ATM and CHK2) to phophorylate p53 at serine residues 15 and 20 respectively. This alteration then induces the p53 mediated transcription of a number of other mediators to induce either cell cycle arrest or apoptosis. Where p53 is mutated, this effect is lost and hence the cellular response to radiation is altered. This pathway is summarised in figure 5-1

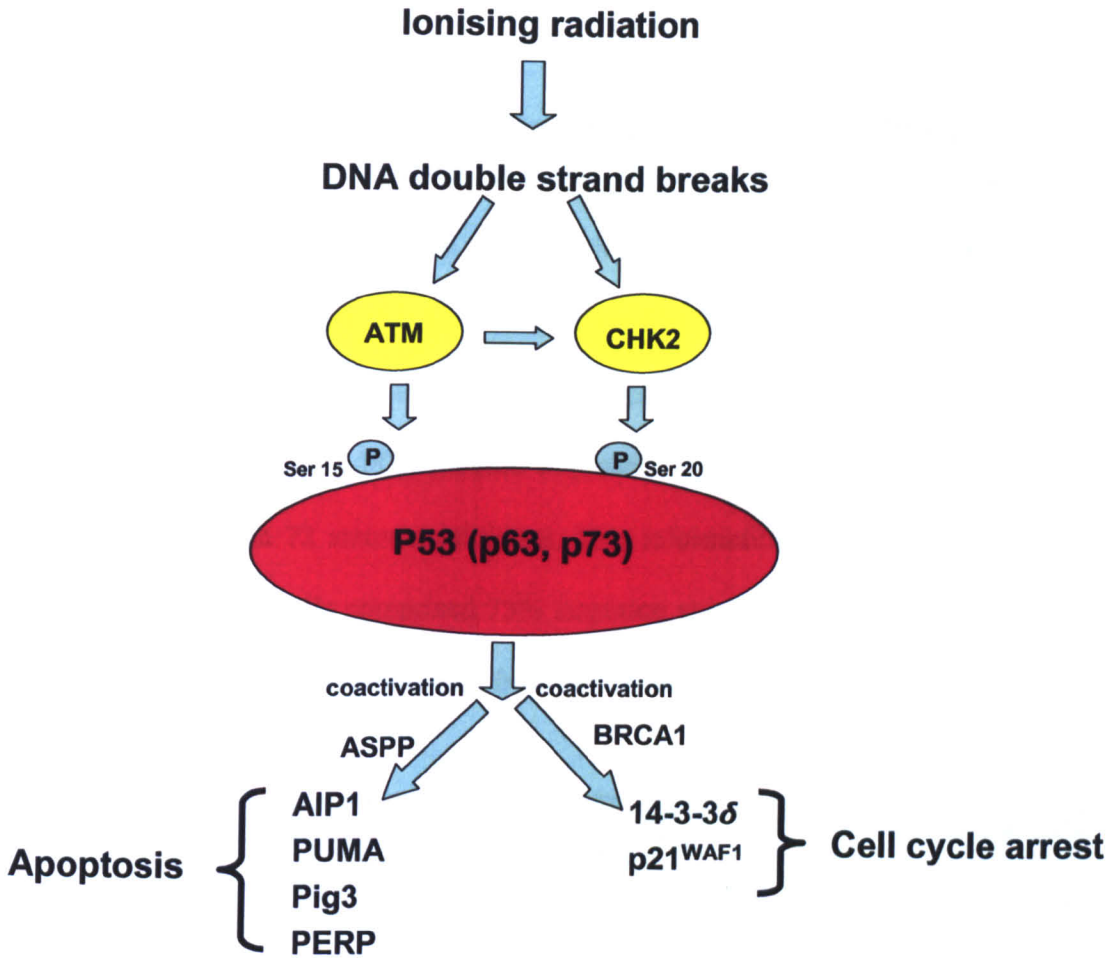


Figure 5-1. Cellular response to ionising radiation mediated by phosphorylation of p53 protein family members

The clear implication from work on this polymorphism is that the while in the absence of wild type p53 the other p53 family members can continue to exert p53-like tumour suppressive effects, the mutated Arg 72 p53 protein may be capable of interfering with these protective pathways. We therefore set out to establish the codon 72 status in our cell lines and whether the oncogenic effect of mutated p53 Arg 72 could be demonstrated in the context of the cellular response to ionising radiation by examining radiation survival at 2Gy and 4Gy in a number of SCCHN *in vivo* derived cell lines.

5.2. Results

5.2.1. Establishing the codon 72 status of SCCHN cell lines.

In total 15 cells lines were analysed for codon 72 status and radiation survival at 2Gy and 4Gy. Eleven of these were derived from *in vivo* SCCHN from Scottish patients and four others were obtained from the American tissue Culture Collection. Genomic DNA was harvested from cells grown in tissue culture as described in chapter 2. This was quantified and then amplified using a PCR method to provide exon 4 DNA. This was sequenced in the BICR and codon 72 status established. This information is summarised in table 5.1. The Scottish derived cells comprised 73% Arginine at codon 72 and 27% proline. This is shown in figure 5.2. All cells obtained from ATCC are arginine at codon 72.

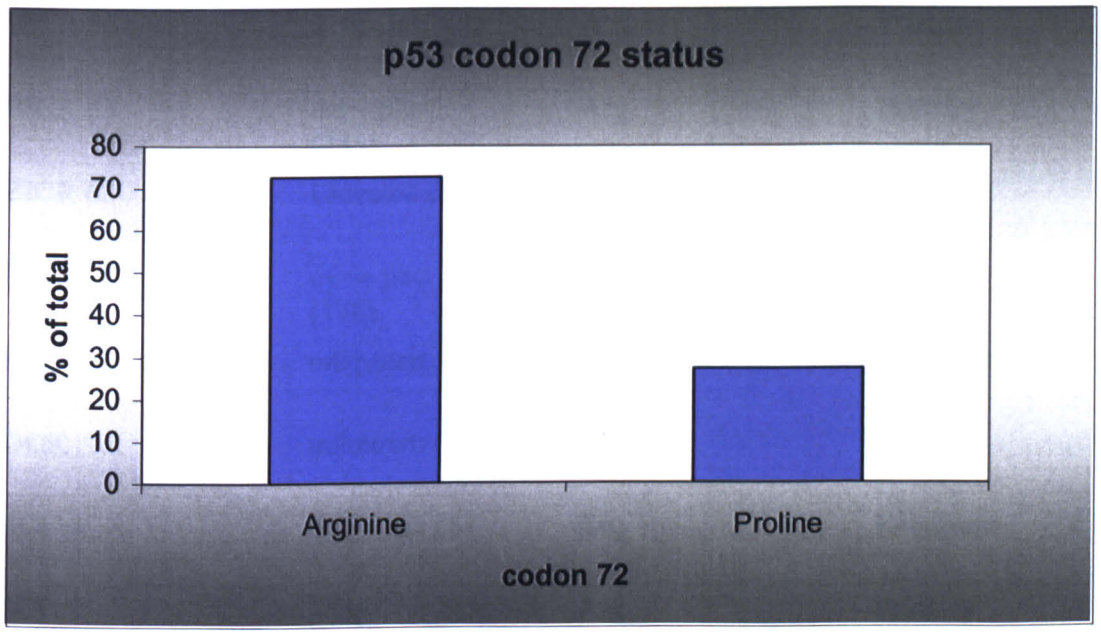


Figure 5-2. codon 72 status in SCCHN cell lines

p53 codon 72 status in SCCHN cell lines derived from *in vivo* SCCHN tumours in Scottish patients at the Beatson Institute for Cancer Research

Table 8. Codon 72 status, p53 mutation and primary or recurrent status of SCCHN cell lines

p53 mutation status and codon 72 status in cells assessed for radiation survival and anaphase bridge scoring. Information was acquired from Burns *et al* (Burns, Baird et al. 1993) and experimentally as described in chapter 2

SCCHN Cell line	P53 status	Codon 72 status	Tumour of origin status (primary or recurrent)
BICR 3	arg→ pro (282)	Arg	Primary
BICR 6	glu→ stop (192)	Arg	Primary
BICR 10	No protein	Pro	Recurrent
BICR 16	trp→ stop (146)	Pro	Recurrent
BICR 31	3bp del	Arg	Primary
BICR 56	21bp del	Arg	Primary
BICR 63	iso→ phe (255)	Arg	Primary
BICR 68	Elevated p53	Arg	Primary
BICR 78	cys→ phe (176)	Arg	Primary
BICR 82	unknown	Arg	Recurrent
DOK	unknown	Pro	Primary
SCC 4	pro→ ser 151	Arg	Recurrent
SCC 9	32bp deletion; 285+ out of frame	Arg	Primary
SCC15	deletion	Arg	Primary
SCC 25	2bp deletion; 209+ out of frame	Arg	Primary

5.2.2. Radiation survival data for SCCHN lines at 2Gy and 4Gy.

Each of the 15 cell lines was grown in tissue culture, split into suspension, irradiated, replated and cultured to obtain countable colonies as described in chapter two. All experiments were once again repeated in triplicate to obtain the data shown in figure 5-3.

This data demonstrates a range of radiation survival across the cell lines tested at 2 Gy and 4 Gy. The figure also shows the range for SF2 for normal keratinocytes. It is clear that SCCHN lines are generally more radioresistant than non malignant cells. The single exception here is BICR7 which, as described in detail in chapter 3 is an unusual cell line in terms of both its telomere function and radioresistance.

5.2.3. Radiation survival data with codon 72 Arg/ Pro status.

For the hypothesis that mutated p53 Arg72 to be true, clearly cells require to have p53 protein translation occurring. We therefore have divided the data into those lines with gain of function and those with loss of function mutations of p53. For each of these groups these are further divided according to codon 72 status.

Figure 5-4. shows radiation survival at 2 Gy for cell lines with gain of function p53 mutation comparing Arg 72 with Pro 72. Only a single gain of function Pro 72 cell line was available (DOK) and this is compared with six gain of function Arg 72 lines, the mean SF2 for which is shown. While there is a range of SF2 for Arg 72 the mean value is similar to SF2 for DOK.

In figure 5-5. the same SCCHN cell lines are depicted, this time with the surviving fraction at 4 Gy of radiation. Again a range of values is seen in the Arg 72 lines, the mean value being in fact similar to that for DOK.

Loss of function cell lines are depicted in figures 5-6. and 5-7. In this case two of the lines were pro 72 and as anticipated given the absence of p53 protein there is no significant difference in radiation survival at either 2Gy or 4Gy for either coded polymorphism.

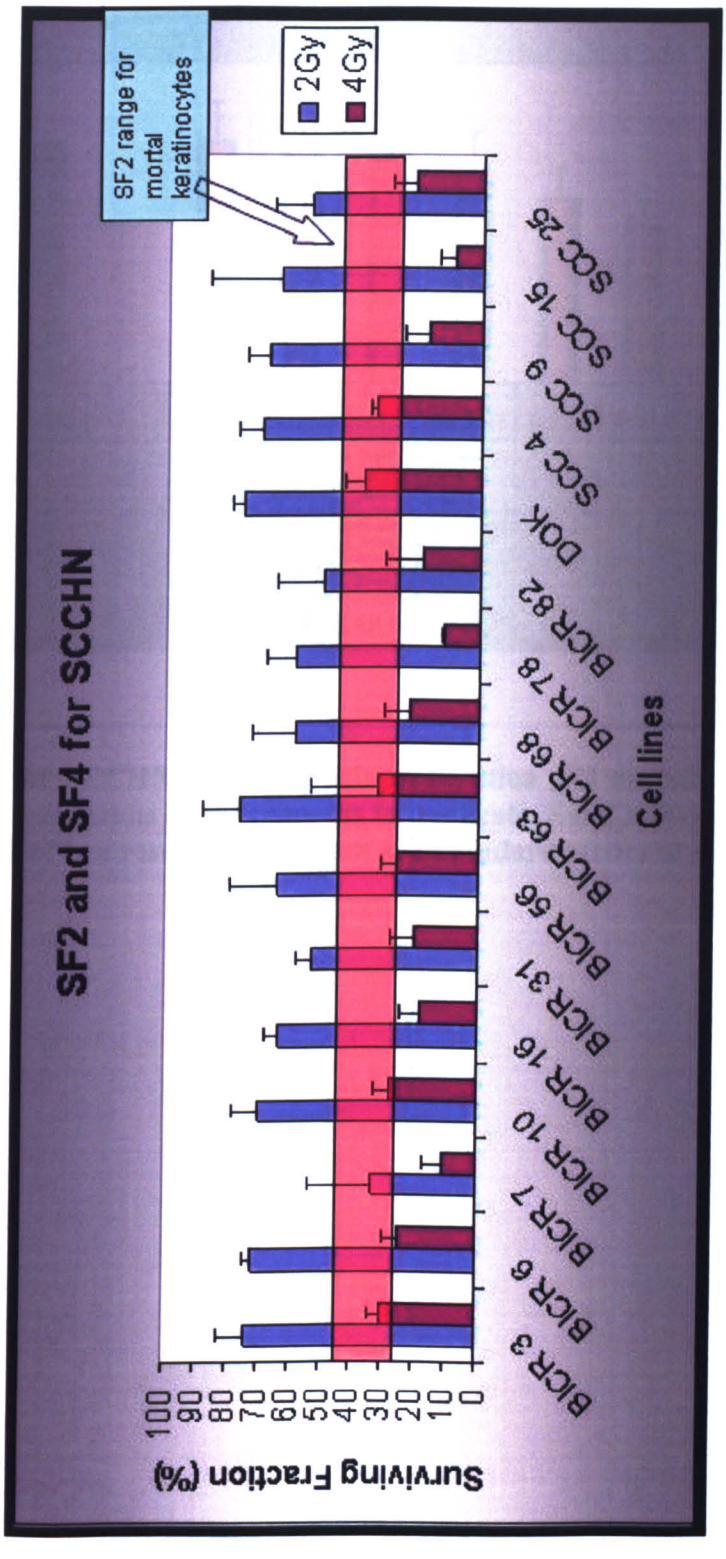


Figure 5-3. SF2 and SF4 for SCCHN cell lines

Surviving fraction at 2Gy and 4Gy is shown for SCCHN cell lines together with range of SF2 for wild type human keratinocytes. Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Error bars are Standard errors of means.

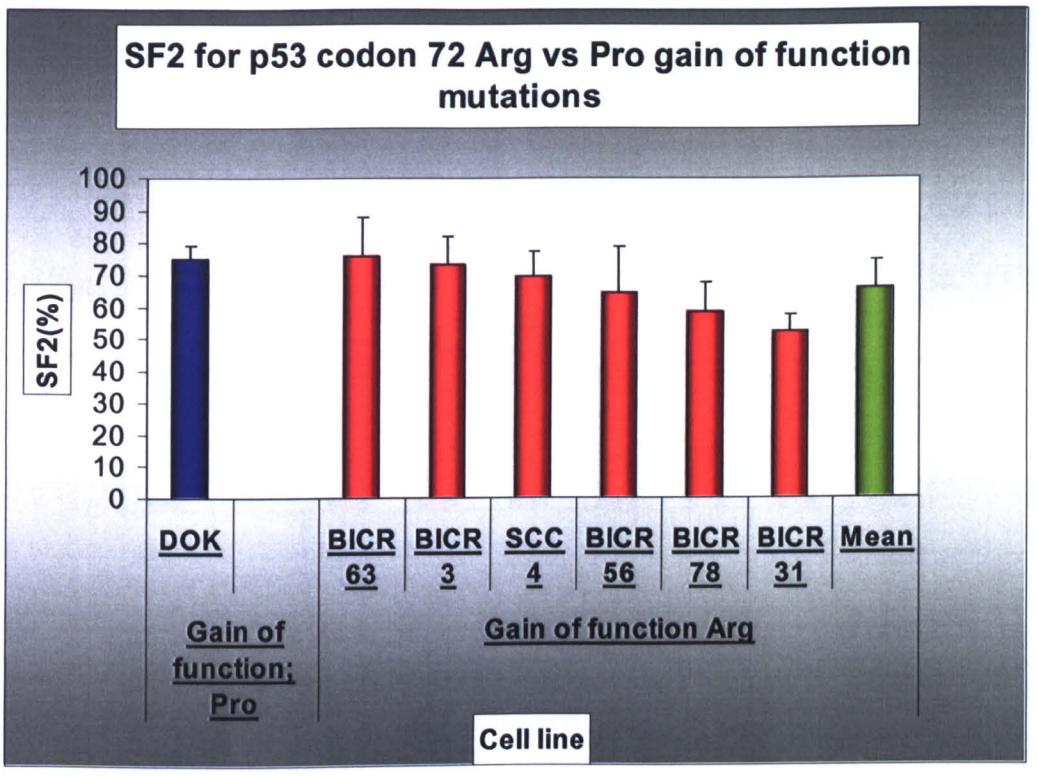


Figure 5-4. SF2 for SCCHN cells with gain of function p53 mutation; Arg 72 vs Pro 72. Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Error bars are Standard errors of means.

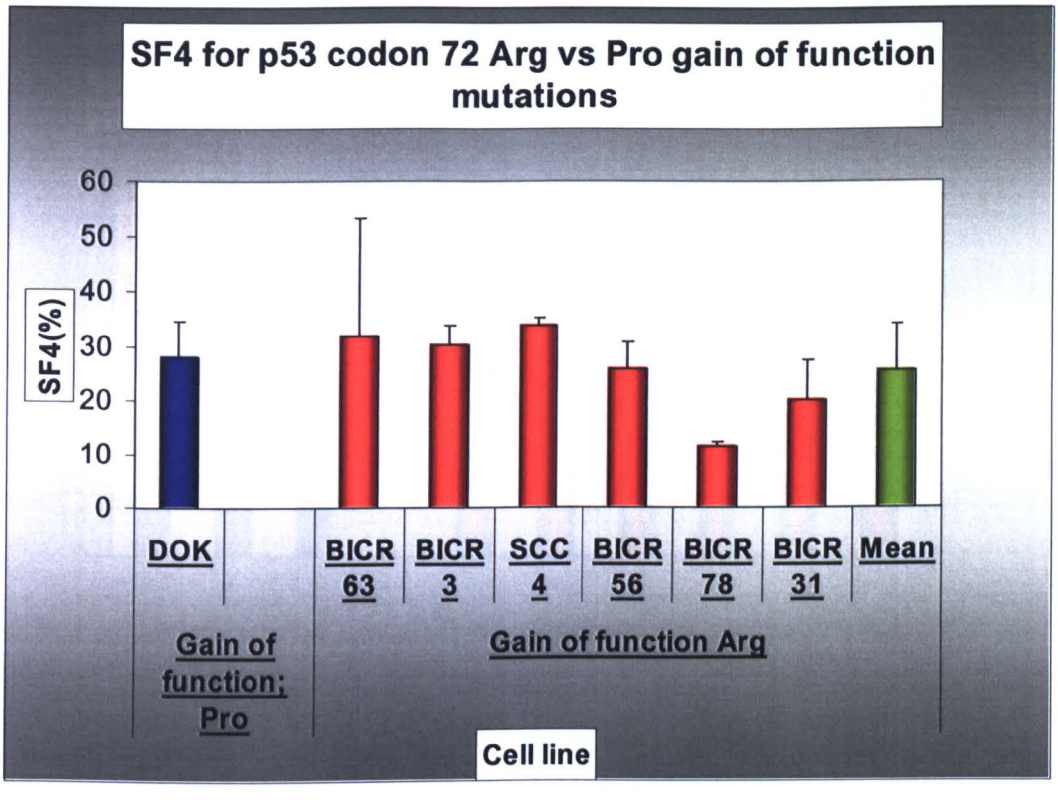


Figure 5-5. SF4 Gy for SCCHN cell lines with gain of function p53 mutation Arg 72 vs Pro 72. Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Error bars are Standard errors of means.

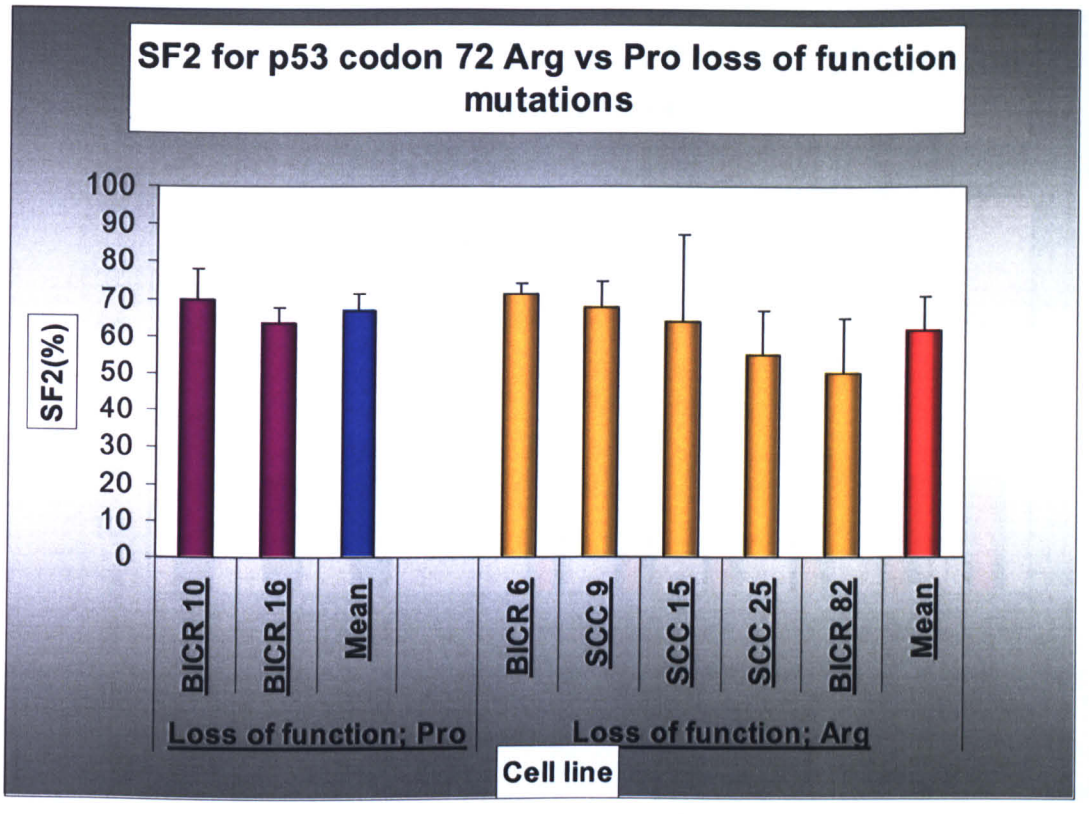


Figure 5-6. SF2 for SCCHN cell lines with loss of function p53 mutation Arg 72 vs Pro 72. Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Error bars are Standard errors of means.

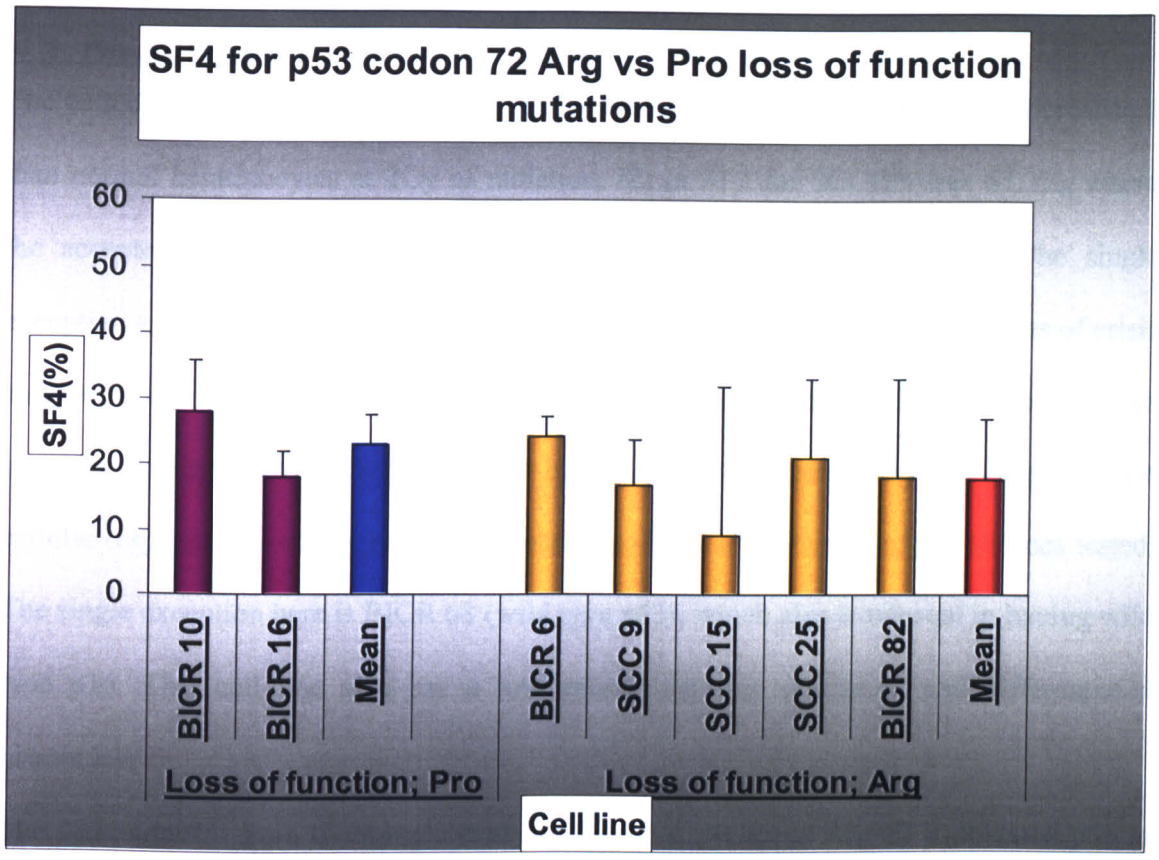


Figure 5-7. SF4 for SCCHN cell lines with loss of function p53 mutation Arg 72 versus Pro 72 Values represent means of experiments in triplicate. Each individual experimental value is the mean of two results. Error bars are Standard errors of means.

5.3. Discussion.

The data obtained from these experiments show that SCCHN cells are more radioresistant than normal keratinocytes at 2Gy of radiation. Mean SF2 for SCCHN was 67.3%, where the accepted range of SF2 for non-malignant keratinocytes is 25-45%. The single exception to this was BICR 7 (SF2=33.3%), a cell line which shows many features of crisis and expresses low levels of telomerase. (Gordon, Ireland et al. 2003) The basis of this increased radiosensitivity may lie at least in part in the understood key role of p53 in the cellular response to ionising radiation. Notably p53 is mutated in 15 of the 16 lines tested. The single exception here is BICR 68 (wild type p53), which also is unusual in having wild type p16. This cell line also has a low colony forming efficiency and differentiated phenotype.

The data obtained from the sample analysed show an excess of Arg 72 in mutated p53 in cancer in the SCCHN cells. This does conform to the hypothesis of excess of this polymorphism in the population, but is at a higher than expected proportion (73% Arg 72) thus perhaps reflecting preferential loss of the Pro 72 allele in heterozygotes (Brooks, Tidy et al. 2000). It is important however to interpret such observational data carefully. It must be considered that this is a small sample and so firm conclusions based on observational data cannot be drawn.

Several of the lines analysed had loss of function p53 mutations and so this short series is not consistent with the hypothesis of Crook et al which suggests that 90% of p53 mutations in SCCHN are gain of function. (Gasco and Crook 2003) Only seven of the lines tested were gain of function mutations and were therefore appropriate for analysis in assessing any potential oncogenic effect of mutated p53 Arg 72 versus Pro 72. The data obtained do not show any difference in radiosensitivity between these groups. Loss of function p53 mutations were also analysed as control data and these too show, as expected, no difference in radiosensitivity between either polymorphism.

In conclusion, the data presented are unable to show any significant difference in the response to ionising radiation of SCCHN cells based on p53 codon72 arginine or proline

Chapter 6

Telomere function and radiation survival in SCCHN cell lines.

6.1. Introduction.

6.1.1. Telomere function.

Telomere function requires adequate length of telomeric DNA repeats and other telomere components to allow appropriate capping. (Blackburn 2001) Where these are lacking and telomeres are dysfunctional, chromosome ends fuse and these can be identified within the dividing cell. The resulting breakage-fusion-bridge events are considered to be a major contributor to the high levels of rearrangement of genetic material observed in the malignant cell. Additionally, shortened telomeres have been shown to impart radiosensitivity syndrome in telomerase null mice and increased radiosensitivity is observed in tumour cell lines with shorter telomeres.

6.1.2. Assessment of Telomere Function.

Telomere function can be assessed by analysis of phenotypic features. Mitotic figures can be examined for evidence of telomere dysfunction and some of the phases of mitosis are demonstrated in figure 6-1. Evidence of fusion of the ends of daughter chromosomes presents as bridged anaphase mitoses (Figure 6-2.) An anaphase bridge is defined as an anaphase mitosis where 2/3 of the distance between the separating poles is spanned by a bridge of two fused chromosomes. (Rudolph, Millard et al. 2001; Gordon, Ireland et al. 2003) The proportion of anaphase mitoses which are bridged due to this fusion of daughter chromosomes with inadequate telomere function can be expressed as a ratio of the total number of anaphase mitoses seen in a tumour cell population. This is termed the anaphase bridge index (ABI) (Gordon, Ireland et al. 2003).

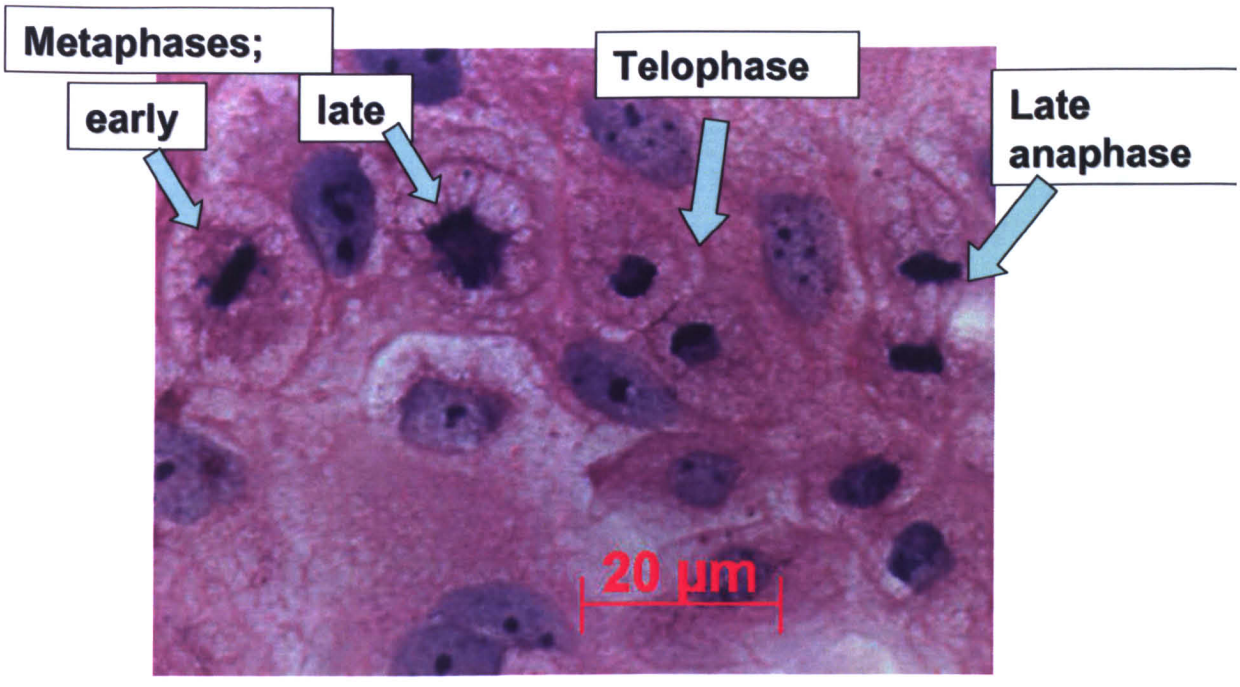


Figure 6-1. Mitotic figures seen in a H&E stained slide culture of BICR 10

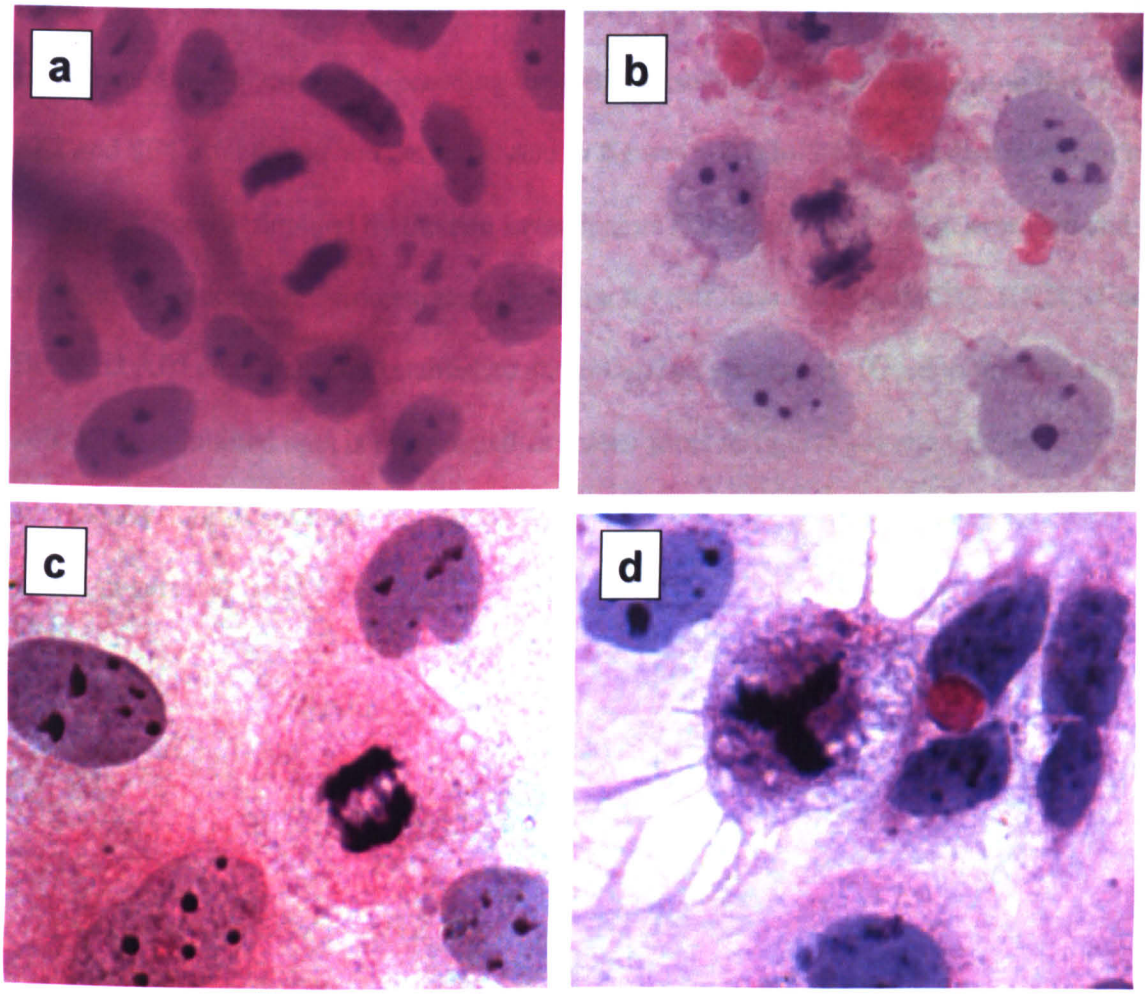


Figure 6-2. Normal and abnormal anaphase mitoses in SCCHN cell lines

H&E stained culture slides showing anaphase mitoses and a tripolar mitosis; a. A normal anaphase mitosis in SCC9. b. A bridged anaphase mitosis from BICR3. c. A bridged anaphase mitosis from BICR 56 and d. a tripolar mitosis seen in BICR78

Previous studies imply that where telomere dysfunction is observed this may reflect either insufficient length of telomeric DNA or other structural defect. (Blackburn 2000)Given the localisation of DNA double strand break repair proteins bound at the telomere reduced telomere function could be speculated to be associated with reduced radiation survival. We investigated this question by recording the ABI for each cell line and comparing this to the SF2 and SF4 established and demonstrated in figures 5.4.1. to 5.4.4. ABI data is shown in figure 6.3. Scores obtained by the two investigators (JMcC and EKP) were averaged and standard deviations are demonstrated. Note that for cell lines BICR6, BICR7, BICR 31 and BICR 63 ABI data had been established and published previously. (Gordon, Ireland et al. 2003) The combined data is represented as scatter plots in figures 6.4 and 6.5. No statistically significant linear regression (ABI = $\alpha + \beta$ x SF[2 or 4]) was found for either radiation survival point.

6.2. ABI and Radiation Survival.

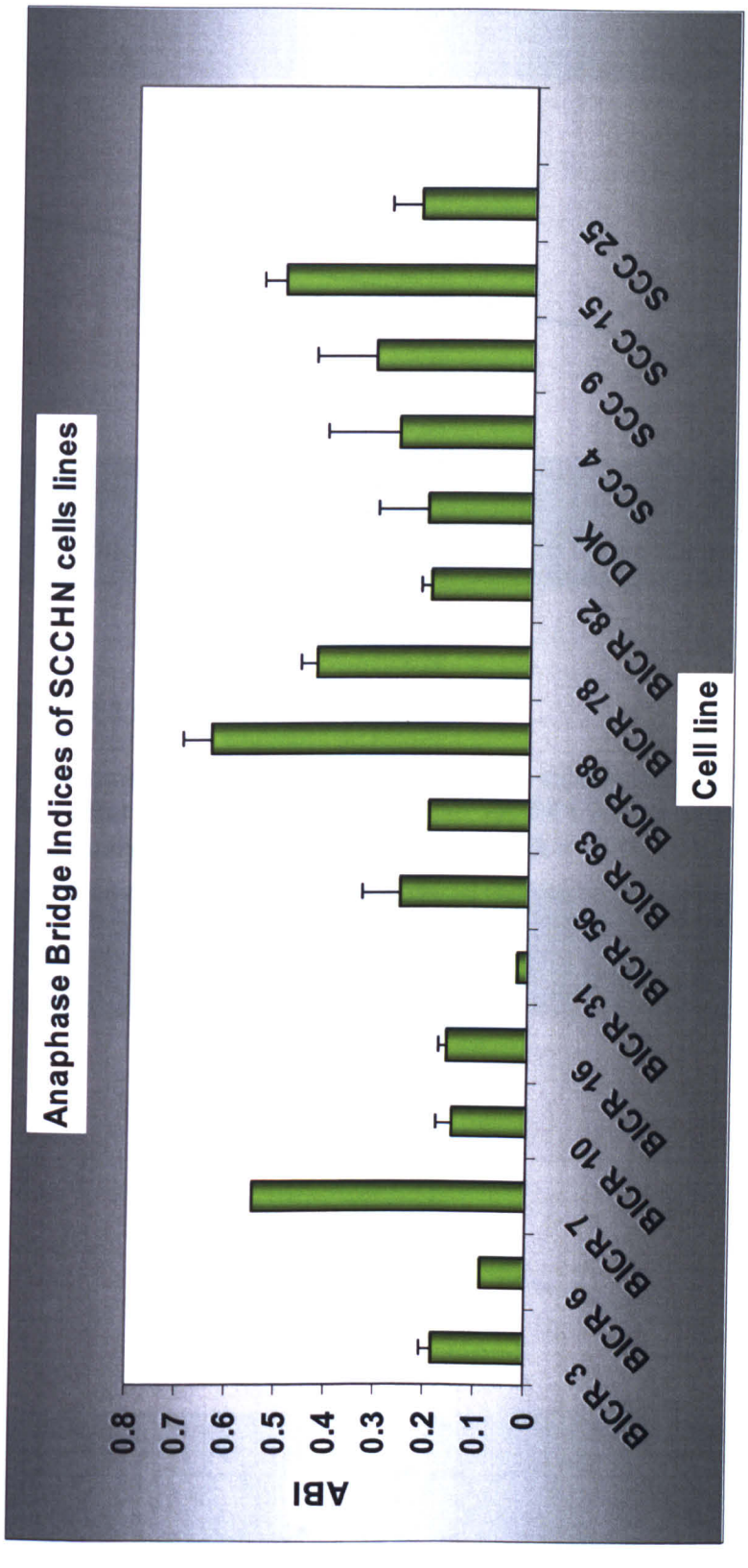


Figure 6-3. Anaphase bridge indices for the 16 SCCHN cell lines examined. Values are averages, bars represent individual values from the two observers.

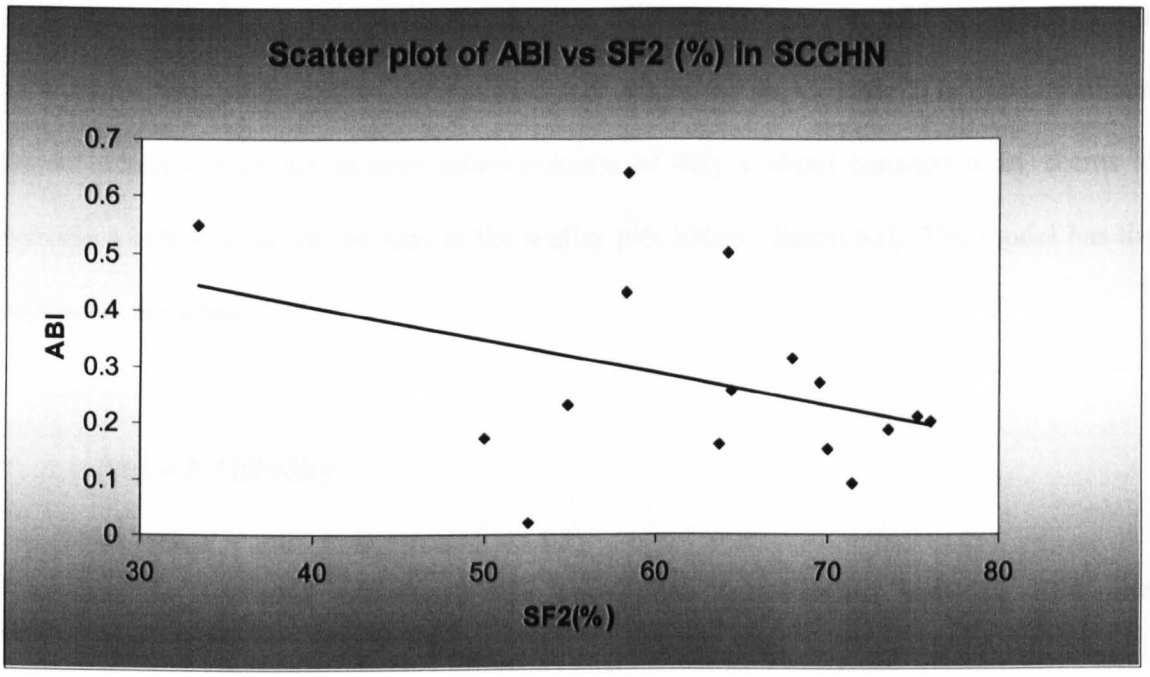


Figure 6-4. ABI vs SF2 for SCCHN cell lines

This scatter plot demonstrates the lack of any linear relationship between ABI and surviving fraction after 2Gy of ionising radiation for SCCHN. A trend line has been added. Regression analysis shows Pearson correlation R = -0.38, p=0.146.

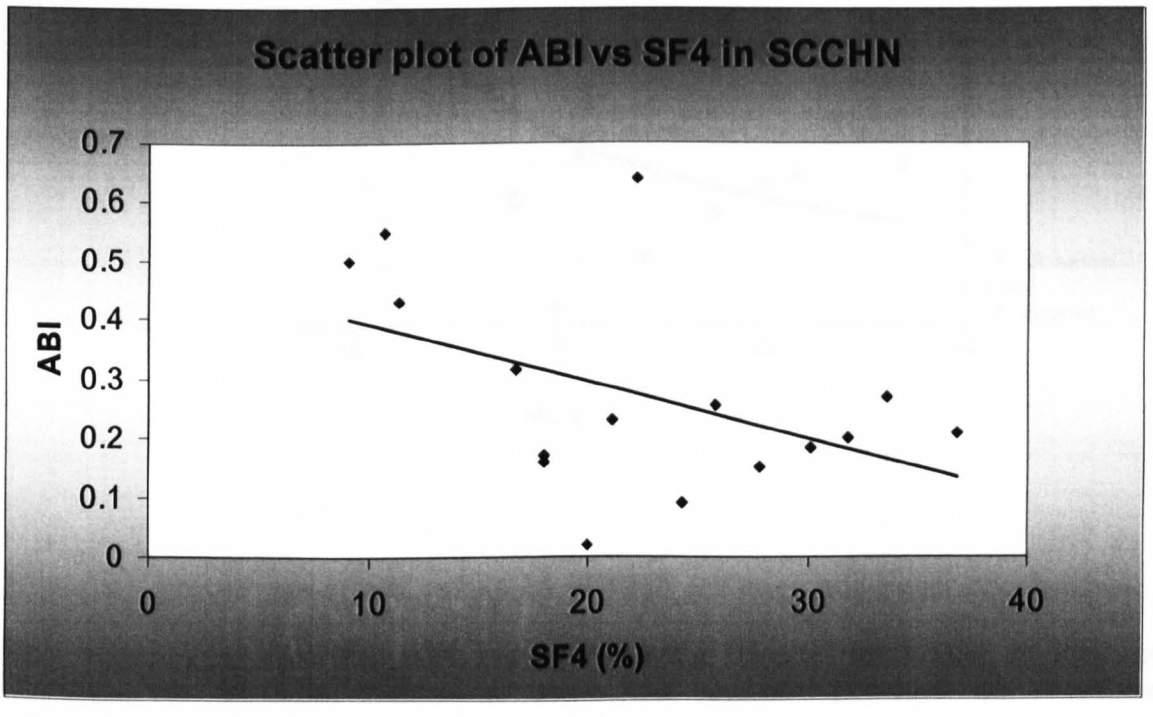


Figure 6-5. Scatter plot of ABI vs SF4 for SCCHN cell lines

This scatter plot demonstrates the relationship of ABI and surviving fraction after 4Gy of ionising radiation for SCCHN.and shows the lack of any linear relationship. A trend line has been added. Linear regression analysis (ABI= α + β x 4Gy) shows Pearson correlation = -0.47, p=0.067.

In further analysing a possible model for the relationship between ABI and the SF2 and SF4 values, non-linear regression was assessed. While for the variable 2Gy non significant results where found, the inverse transformation of 4Gy without constant term, seems to provide a better fit as can be seen in the scatter plot below (figure 6.6). The model has the following equation:

ABI = 5.107/4Gy

(p value < 0.001; R square =0.82)

Equation 3. Formula describing the non-linear relationship between SF4 and telomere function assessed by ABI.

Figure 6-6. Scatter plot of ABI vs SF4 for SCCHN cell lines; non-linear relationship.

This scatter plot describes ABI versus surviving fraction after 4Gy of ionising radiation for SCCHN. Non-linear regression analysis provides the equation ABI=5.107/SF4(%). R square = 0.82, p<0.001.

6.3. Discussion.

Attempts to establish a correlation between telomere function by anaphase bridge score and radiation survival produced interesting results. No correlation of significance was found between ABI and SF2. For ABI and SF4 a clear, non-linear correlation was demonstrated ($p<0.0001$, R square = 0.82). This provides further support for the role of the telomere in radiosensitivity; deteriorating telomere function produces increased cell death on exposure to ionizing radiation in a predictable manner. The lack of correlation at 2Gy is an interesting finding. This radiation dose corresponds to a single fraction used therapeutically and would be anticipated to induce around 300,000 ionisation events in the nucleus and a wide range of DNA damage but only approximately two DSBs per chromosome. Most DNA damage would be repaired within two hours of exposure. An approximate doubling of each of these values is expected after 4Gy exposure and at this dose we see a tight correlation with radiation survival. Tumour response to radiotherapy is known to be a multifactorial phenomenon involving such factors as cellular oxygenation, tumour doubling time and bystander effects. These experiments control for oxygenation with constant incubator conditions and for bystander effect by the use of single cell suspensions. It may simply be the case that for SCCHN a single 2Gy fraction is insufficient to elaborate the true radiosensitivity of the cancer cell.

Further work is now needed to establish whether this finding translates into clinical responses to radiotherapy. Analysis of ABI at tumour diagnosis and analysis of any correlation with survival will be not only of interest but of relevance in determining prognosis. Should this also prove to be useful information then any changes in ABI following therapeutic use of an antitelomerase agent may provide an index of likely success of radiotherapy or other double strand break inducing agent. The data obtained suggest that increasing ABI from 0.2 to 0.4 could reduce SF4 from ~25% to less than 10%. Clearly, the biological nature of the systems involved in all these processes produce

variation and so as with almost all prognostic parameters ABI will predict across a population rather than for the individual.

Chapter 7.

Results.

Telomere function and radiation survival in Primary and Recurrent SCCHN; Analysis of SCCHN Cell lines and archival SCCHN tumour sections.

7.1. Introduction.

Tumour progression refers to the selection process which occurs within the tumour cell population during the lifespan of a cancer. This selective pressure continues to produce clones of cells which are more suited to the environmental stresses placed upon them. These stresses include the cytotoxic effects of therapeutic agents, including chemotherapy and radiotherapy.

This concept is particularly relevant in the context of recurrent malignancy. Clearly tumour progression has been ongoing in this circumstance and the tumour population which persists has escaped therapeutic attempts at cure, which in the context of SCCHN will often have included radiotherapy.

We therefore sought to establish whether the radioresistance of recurrent SCCHN differed from that of primary tumours by assessing radiation survival at 2Gy and 4Gy in cell lines derived from tumours in these two categories.

Tumour progression might also be predicted to select for enhanced telomere function, which is mediated by telomerase upregulation in SCCHN. If this occurs, then an antitelomerase therapeutic strategy may be less effective in the recurrent disease scenario. Since any new therapeutic agent will inevitably go through phase I and II testing in the context of advanced cancer, it may be that antitumour effects are less marked here than would be the case for early disease. We therefore assessed telomere function in primary and recurrent disease by ABI scoring of archival sections of primary SCCHN tumours and recurrences in the same patients.

7.2. Primary versus recurrent status and radiation survival in SCCHN cell lines.

Using the panel of cell lines assessed in this investigation we were able to compare cells derived from primary lesions with those from recurrent SCCHN. Each of the recurrent SCCHN assessed had been previously treated and this information is contained in table 7.1. Here we attempt to provide initial data regarding any change in radiosensitivity in recurrent tumours which have failed standard therapy.

Table 9. Therapeutic modalities previously utilised in management of four recurrent SCCHN cell lines

Recurrent cell line	Surgery	Radiotherapy	Chemotherapy
BICR 10	Yes	Yes	Yes
BICR 16	Yes	Yes	Yes
BICR 82	Yes	Yes	Yes
SCC 4	Yes	Yes	Yes

Radiation survival data is shown in figure 7.1. Here the mean values for SF2 are very similar for primary versus recurrent malignancy (primary 62.5%, recurrent 63.3%). For survival after 4Gy similarly there is little difference (primary 21.7%, recurrent 24.4%). Although the survival values are consistently greater for recurrent disease, the differences are not statistically significant (SF2; $p = 0.9$, SF4; $p = 0.6$).

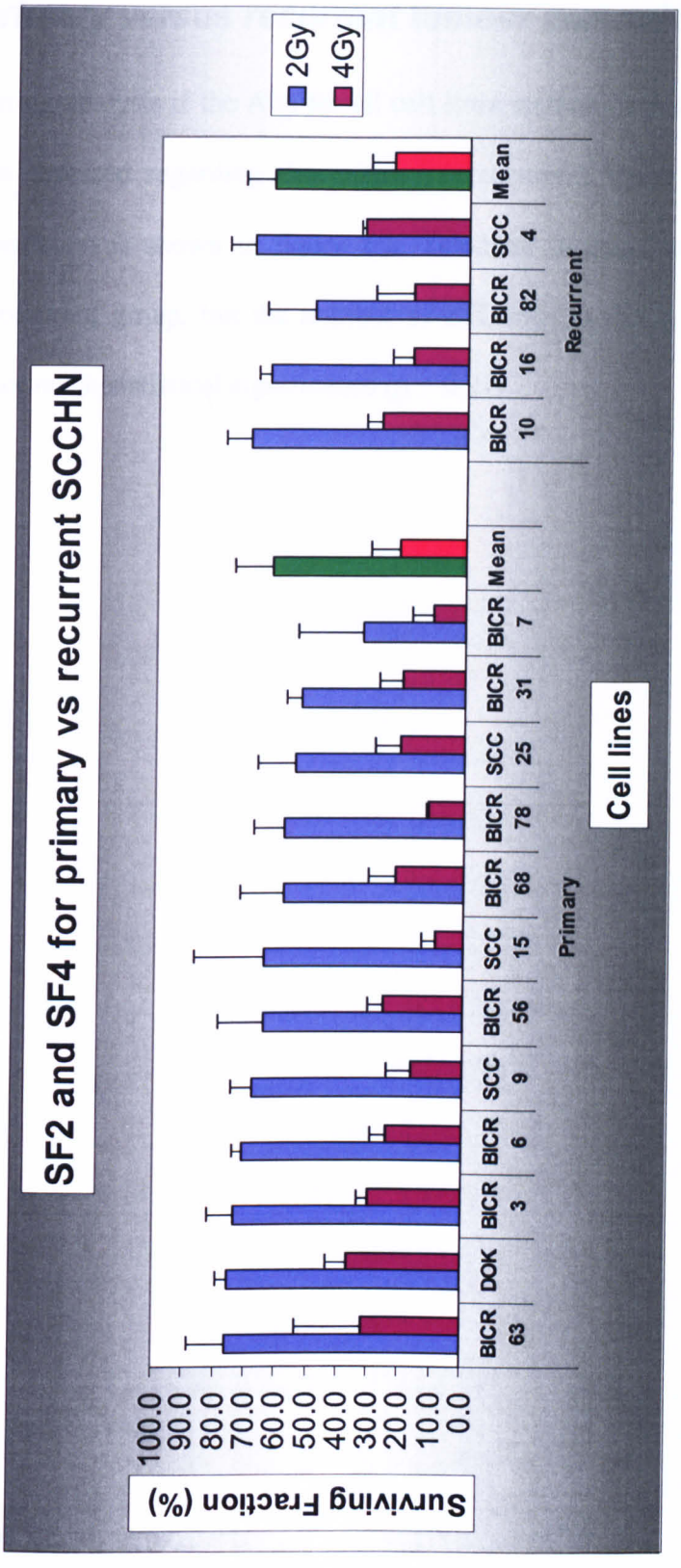


Figure 7-1. Radiation survival at 2Gy and 4Gy comparing primary and recurrent SCCHN cell lines. Values represent means of experiments carried out in triplicate, each experiment carries duplicate plates. SF2 and SF4 are demonstrated for SCCHN derived from primary (BICR63 to BICR7) and recurrent (BICR10 to SCC4) tumours with mean values for each group. Mean differences are not statistically significant (SF2 p= 0.9, SF4 p = 0.6)

7.3. ABI in primary versus recurrent tumour derived SCCHN cell lines.

Data obtained during analysis if the ABI for all cell lines studied during the course of this investigation was assessed regarding the primary or recurrent status of the tumour of origin. This information is shown in figure 7-2. Telomere function assessed by ABI is superior in the recurrent group, but the number of cell lines in this group is small. The difference does not reach statistical significance ($p = 0.21$).

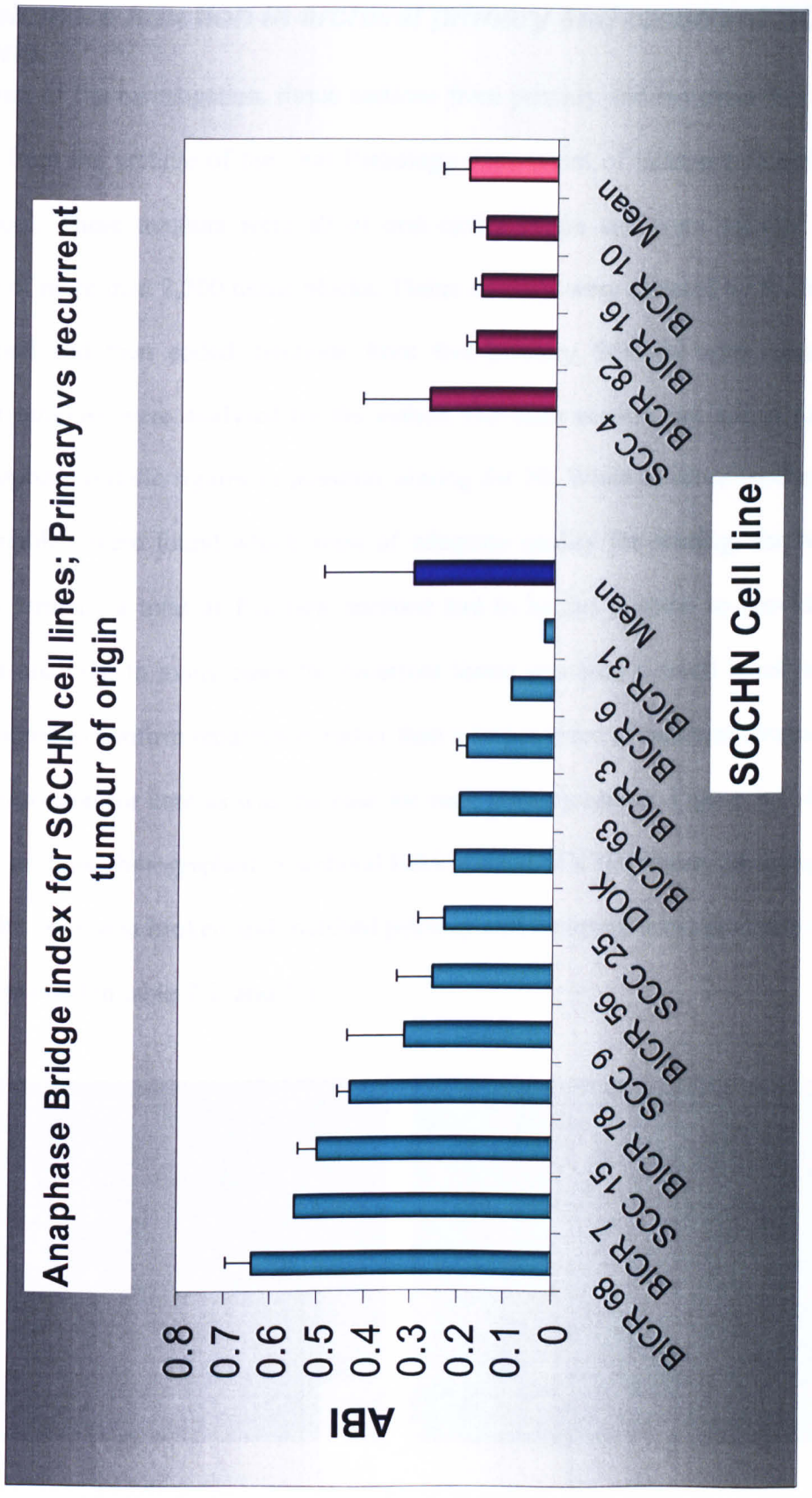


Figure 7-2. ABI for primary and recurrent SCCHN cell lines. ABI is demonstrated for SCCHN cell lines derived from primary (BICR 68 to BICR 31) and recurrent (BICR 16 to SCC4) with mean values. Each value is the mean of two observer scores indicated by the error bars. Differences do not reach statistical significance (p = 0.21)

7.4. Telomere function in archival primary and recurrent SCCHN sections.

In this part of the investigation, tissue sections from primary and recurrent SCCHN were selected from the archive of the Oral Pathology department of Glasgow Dental Hospital and School. These tumours were all of oral cavity origin and were selected from the database of more than 2,500 tissue blocks. Tissue sections were selected by Professor D G MacDonald and then coded. Sections from five primary SCCHN with corresponding recurrent tumours were analysed by the author. For each section, we aimed to score as many anaphase mitotic figures as possible, aiming for 20. While in some sections a much greater number were found which were of adequate quality for scoring, for two of the recurrent tumours a total of five new sections had to be cut in order to provide enough tissue for analysis. In many cases the recurrent tissue was only a small block which had been obtained to confirm recurrence, rather than a larger piece of material obtained during surgical resection for cure as was the case for each primary cancer. Figure 7-1 shows two anaphase mitoses photographed in archival H&E stained SCCHN tissue. On completion of scoring the code was broken and matched primary and recurrent tumours compared. This data is contained in table 7-2. and 7-3.

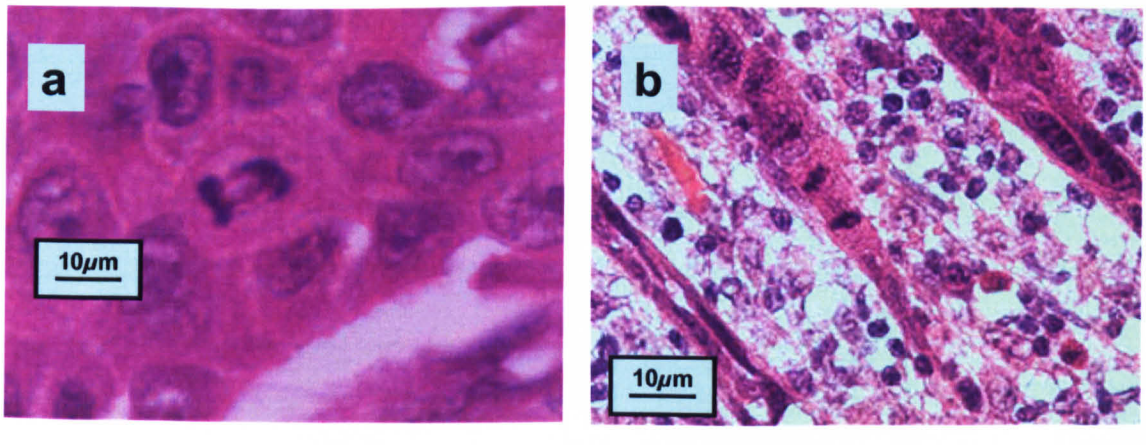


Figure 7-3. H & E stained tissue sections from SCCHN tumours

This figure illustrates a. bridged anaphase mitotic figure. b. normal anaphase mitosis in an invading cord of tumour cells.

Table 10. ABI for five cases of primary and corresponding recurrent SCCHN scored under light microscopy

	anaphases scored	ABI	Time to recurrence.
Case 1	23	Primary 0.44	28 months
	29	Recurrent 0.32	
Case 2	67	Primary 0.6	12 months
	15	Recurrent 0.4	
Case 3	30	Primary 0.6	45 months
	37	Recurrent 0.28	
Case 4	72	Primary 0.3	14 months
	32	Recurrent 0.34	
Case 5	106	Primary 0.33	12 months
	130	Recurrent 0.33	

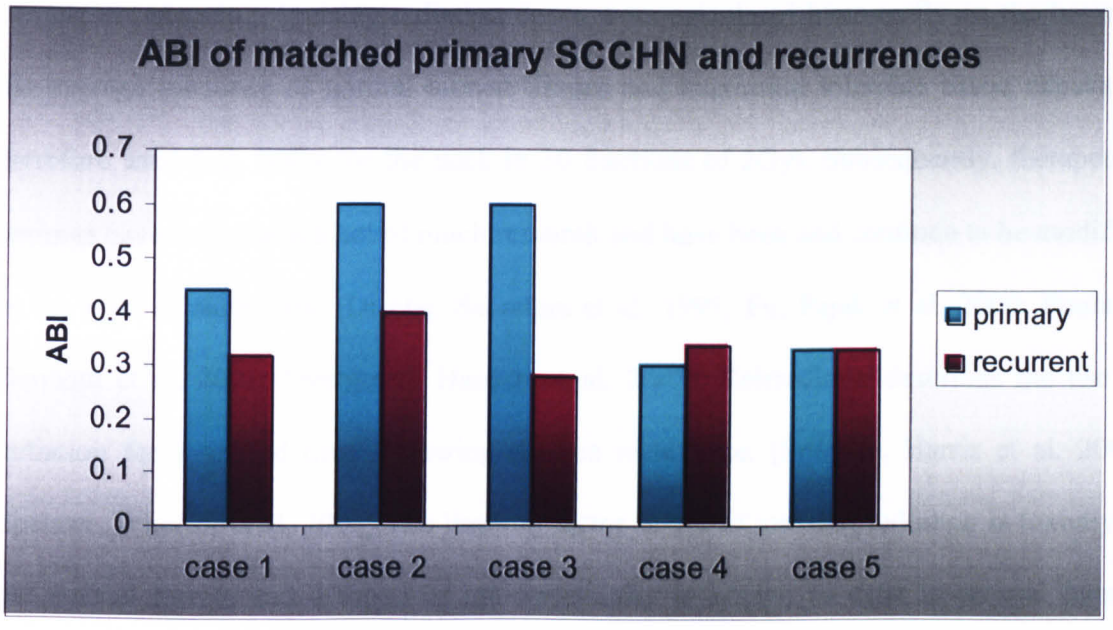


Figure 7-4. ABI of matched SCCHN primary and recurrent tumours

In each case either ABI starts low (mean value 0.31) or drops to a lower value in recurrent disease (mean value 0.33), indicating improved telomere function in recurrent disease.

7.5. Discussion

In tumour tissue where disease recurs after therapeutic intervention, the tumour progression process has continued for many months. If management is surgery only, recurrence from microscopic residual tumour deposits represents a cell population which has undergone clonal evolution for some time. Where chemotherapy and or radiotherapy have been used additionally, there has clearly been additional selective pressure in the tumour microenvironment. All three recurrent SCCHN cell lines studied here were derived from tumours where chemotherapy and radiotherapy had been used therapeutically and so had been subject to exactly this pressure.

Cells derived from recurrent SCCHN tumours were not significantly different in radioresistance than those derived from primary cancers. This may indicate that for SCCHN derived from the Scottish population, ionising radiation may still be of value in the context of disease recurrence and so this novel information may be of value in

therapeutic planning. Ionising radiation doses were calculated historically on the basis of the average tolerance of normal human tissues and maximum tolerable tissue exposures therefore used (e.g. 60Gy for the neck in 30 fractions of 2Gy). Subsequently, therapeutic regimes have been the subject of much research and have been and continue to be modified in the light of such data. (Dische, Saunders et al. 1997; Fu, Pajak et al. 2000; Poulsen, Denham et al. 2001; Overgaard, Hansen et al. 2003). Reirradition describes the use of radiation for a second time following disease recurrence. (Spencer, Harris et al. 2001; Spencer, Wheeler et al. 2003) The limiting factor in use of ionizing radiation is toxicity to the normal tissues and a range of radiosensitivity is known to exist in normal human tissues (Haikonen, Rantanen et al. 2003) A tumour specific radiosensitiser is an attractive prospect in this context in particular. If telomerase inhibition could selectively radiosensitise SCCHN this would offer the prospect of effective cancer therapy, while limiting host toxicity.

The evidence presented here showing no increase in radioresistance in recurrent SCCHN may provide some support for this strategy. The data series is however small (four recurrent cancers) and so should be interpreted with care.

Telomere function in the recurrent lines shows a difference from that in cell lines derived from primary tumours. While this may point toward improved telomere function in recurrent disease the small numbers of recurrent lines are small and the data does not reach statistical significance.

Tumour tissue sections examined for ABI show some interesting data. Difficulties with this approach include the relative lack of available tissue from recurrent disease. Surgical resection of disease in this context is rarely a viable option and so such tumour specimens are relatively rare and generally of small volume. Further, in some specimens there is a

paucity of mitotic figures, reflecting the replicative state of the constituent clones of tumour cells at the time of fixing of the tumour tissue.

Recurrent disease was identified as further malignant tissue occurring at the site of previously resected tumour tissue at least six months post treatment (see table 7.1 for time to recurrence in each case). In the context of SCCHN there is clear evidence of mucosal field change and so the possibility of second primary cancers emerging in the same patient rather than a true recurrence of disease cannot be entirely excluded.(Jefferies, Edwards et al. 2001)

The data presented conform to the hypothesis that telomere function enhances with tumour progression unless ABI is already at a low threshold value of around 0.3. This reflects similar findings in the study of cervical metastases from SCCHN oral cavity primary tumours. Here the ABI was shown to drop in cells which had successfully spread to the neck. (Gordon, Ireland et al. 2003) This series is however too small to test for statistical significance.

While the data described in this chapter provide for interesting discussion in the context of telomere function and SCCHN disease recurrence, it is not possible to draw firm conclusions due to the small numbers of cases involved. Further examination of more cases in a similarly designed study would enhance the data presented here. An assessment of ABI in tissue samples and correlation with survival would also be valuable data to assess the significance of telomere function in determining disease outcome. This may be useful both in establishing prognosis and also in quantifying any survival advantage conveyed by antitelomerase therapy induced telomere dysfunction.

Chapter 8.

Discussion.

8.1. Summary of experimental results.

The experimental evidence accumulated in this thesis provides some insight into telomerase manipulation and its effect on telomere function and the impact of this effect on the radiosensitivity of SCCHN. Restoration of high levels of telomerase expression by stable transfection of the hTERT gene in the BICR7 cell line produced increased radioresistance. This was not seen in BICR6 which has already high levels of telomerase and robust telomere function, nor in normal human fibroblasts or the GM847 ALT line.

When telomerase catalytic activity was successfully inhibited by AZT, a deterioration in telomere function was observed. This was accompanied by a dose dependent reduction in radiation survival after exposure to ionising radiation. This effect was ablated by high level telomerase expression in BICR6Tert cells and was not seen in GM847 ALT cells, thus confirming both ablation of the effect with excess telomerase and telomerase specificity of effect.

Radiosensitivity of SCCHN tumours is likely to be a multifactorial phenomenon. Studies on p53 codon 72 polymorphism had indicated that this variation produced differing responses to cisplatin chemotherapy, mediated via effects on p73. Recognising this we considered the possible impact of p53 codon 72 polymorphism on the response to ionising radiation. The data obtained do not allow us to draw any firm conclusions and a larger series of tumours would be necessary to establish any meaningful correlation.

We discovered a significant non-linear correlation between observed telomere and radiation survival. This further supports the telomere function and radiation survival hypothesis and may indicate that observed telomere function could be used to predict tumour response to radiotherapy. This would be particularly useful in the context of clinical trials of telomerase inhibitor agents. Further data examining telomere function in

recurrent SCCHN tissue and comparing with primary disease implied that telomere function improves with disease progression. These data did not reach significance.

8.2. Final Discussion

In interpreting the experimental data contained in this thesis a number of issues are relevant in consideration of the possible mechanisms involved and in planning further work moving toward telomere function manipulation as a potential therapeutic strategy.

8.2.1. The role of Telomerase in the malignant cell.

The wider issue of the role of telomerase in mammalian cells beyond telomere lengthening and maintenance has received considerable attention. Forced expression of telomerase in both cells and mice with normal length telomeres has shown growth and survival effects of telomerase which are distinct from net telomere lengthening. (Blasco 2002) In the context of exposure to ionising radiation, recent reports have examined the effect of telomerase on normal human fibroblasts. There was no change in SF2 for HFFs ectopically expressing hTERT in the experiments included in this thesis and this has now been confirmed by other investigators. (Ojima, Hamano et al. 2004)

Human fibroblasts immortalised by ectopic telomerase expression have also been shown to have increased spontaneous and post irradiation chromosomal stability. (Pirzio, Freulet-Marriere et al. 2004) This data refers to chromosomal fragmentation seen both in first generation metaphases after irradiation and also some time after irradiation therefore showing that telomerase at these levels in this context confers resistance to both the immediate and late effects of ionising radiation exposure. Further clarity on the role of telomerase in these human cells was provided by experiments showing that the protective role of telomerase was only evident in human fibroblasts with short, presumably near dysfunctional telomeres. Resistance was dependent on the ability of telomerase to elongate short telomeres and was not evident in cells with long telomeres.(Rubio, Davalos et al. 2004)

Ojima *et al* also concluded that ionising radiation tended not to damage telomeres directly, but rather the telomere damage signals were a delayed event after exposure, occurring after multiple cell divisions in the progeny of exposed cells. Of note in all these studies is the level of telomerase expression produced by ectopic expression of the enzyme. This is certainly in excess of that seen in the context of human malignancy and so while this may provide some insight into the interaction of damage mechanisms occurring in normal cells and the overexpressed telomerase enzyme, the relevance of these finding to the post-crisis cancer cell is not clear and firm conclusions cannot, as yet, be drawn. (Gordon, Ireland et al. 2003; Ojima, Hamano et al. 2004; Pirzio, Freulet-Marriere et al. 2004).

Further studies using the mouse model and mouse cells have tended to imply that the telomerase role in radiosensitivity may not be in healing broken chromosome ends, at least not by the addition of telomere repeats. (Latre, Genesca et al. 2004) Telomerase proficient mouse embryonic fibroblasts (MEFs) were compared to telomerase knockout MEFs in response to DSBs induced by exposure to ionising radiation. Telomerase did not affect the efficiency of chromosome rejoining after radiation induced DSBs. Earlier studies by the same group have indicated that telomeric functions and DNA repair pathways are closely linked. In the context of the shortened telomere in late generation mTERC-/- mice, correct repair of double strand breaks is impaired. (Latre, Tusell et al. 2003) Chromosomes with shortened telomeres fused to DSB chromosome ends, interfering with correct repair (see figure 1.6.). This concept is appealing in attempting to explain the increased radiosensitivity seen with age (and accompanying telomere shortening) in humans and may help explain the enhanced radiosensitivity seen in the context of telomerase inhibition (chapter 4) and increased radioresistance in hTERT expressing BICR7Tert cells (chapter 3). The human immortal malignant keratinocyte, however, has a post-senescent and post-crisis telomere equilibrium which relies on telomerase for its maintenance. It is most likely therefore that advances in our understanding of the role of telomerase in radiosensitivity of human cancer cells will come from experiments using *in vitro* human cancer cell models or

indeed correlative information gleaned by scrutiny at the cellular, chromosomal and molecular level during trials of anti-telomerase therapy in cancer. Clearly access to tumour tissue is highly relevant in this context and so SCCHN is an appealing cancer in which to study the potential therapeutic benefits of this approach. This again emphasises one of the intriguing aspects of targeted cancer therapy based on our increasing understanding of the normal and malignant human cell molecular biology. Historically many therapeutic agents were discovered to be useful and the precise basis of this effectiveness established somewhat later, if indeed at all. It is relevant to recall throughout considerations of the potential relevance of these experiments that the exact basis by which ionising radiation is effective in treating malignant disease is unclear. This therapeutic modality shows great variation across tumour types and indeed even within tumour subtypes and even in the same patient.

8.2.2. Telomere binding proteins and telomere function

The topics of telomere binding proteins and their various roles in chromosomal homeostasis are now considered to be extensive and diverse. (de Lange 2002) How these components contribute to telomere functional capping is of relevance to our discussions of the therapeutic application of functional telomere manipulation in cancer and to assays of telomere dysfunction. Regarding the first of these, clearly all normal human cell chromosomes have telomeres and telomere componenets which are normal for that cell type. This means that targeting a telomere binding proteins with capping maintenance function might risk losing the specificity offered by the telomerase targeting strategy.

Where telomere function assays are concerned, recent evidence has shown the existence of a novel telomere cohesion molecule which is cleaved by tankyrase 1. (Dynek and Smith 2004) This acts in the normal cell during mitosis to maintain apposition of the telomere and is cleaved in early anaphase to allow separation of daughter chromosomes. Should this molecule play a role in cancer cell telomere maintenance then this might at least affect anaphase bridging and hence cellular viability and certainly this novel protein merits

further investigation in the cancer cell. In the context of telomerase inhibitor therapy, quantifiable indices of telomere dysfunction are necessary in order to assess the effectiveness of any given agent. The anaphase bridge index is such an assay, but necessarily requires enough mitotic figures visible in any given tumour section to allow quantification. Clearly telomere dysfunction will occur in other cell cycle phases and methods of detecting this would be an improvement on the current situation. Recent reports have described DNA damage foci at dysfunctional telomeres created via inhibition of TRF2. These are associated with response factors such as 53BP1, γ-H2AX, Rad17, ATM and Mre11. These have been termed Telomere Dysfunction-Induced Foci (TIF) and provide the needed means of evaluation of telomere status in cells suspected of harbouring telomere dysfunction. (Takai, Smogorzewska et al. 2003) This method could also be used to corroborate our finding of a non-linear correlation of ABI with radiation survival.

8.2.3. Agents for telomerase inhibition.

While a variety of approaches has been shown to effect inhibition of the telomerase enzyme complex, very few small molecule agents suitable for oral administration have been described. The most novel is BIBR1532 which has shown promise but may be less effective than originally thought. (Barma, Elayadi et al. 2003) Experiments using this agent at higher concentrations in leukaemia cells have produced new information which is of relevance to consideration of how telomerase inhibition might affect the cancer cell. Dose dependant cytotoxitiy was demonstrated in AML and CLL culture assays but not in normal CD34(+) cells. Importantly, cell death did not depend on telomerase catalytic activity, but rather a time dependent telomere erosion effect was seen, cell death being delayed with longer telomeres and associated with loss of TRF2 and p53 phosphorylation. Thus there seemed to be a direct structural damage to the individual telomeres rather than telomerase suppressed telomere shortening, hinting at a loss of some maintenance function of the telomerase enzyme complex at the telomere. (El-Daly, Kull et al. 2004)

In the experiments in chapter 4 we successfully affected telomere function by telomerase inhibition using AZT; one of a number of small molecule reverse transcriptase inhibitor with good bioavailability and well established pharmacological parameters.

Notably, in the context of HIV disease, Kaposis sarcoma is a frequent AIDS related malignancy which is particularly radioresponsive in the context of antiretroviral therapy. An appealing aspect of these experiments is the effectiveness of AZT which is an agent which is already in clinical practice. In these experiments doses of AZT were close to the plasma concentration of drug aimed for in HIV therapy. AZT has been shown to act by competitive inhibition binding to the active site of reverse transcriptase enzymes. It also may be incorporated into growing DNA chains causing either premature chain termination or a mutated telomere which cannot functionally cap. Certainly the short time course (seven days) of our experiments in BICR6 would not allow simple replicative telomere attrition to account for the reduced telomere function and radiosensitivity observed. Incorporation of mutant telomeric DNA has been shown to be an effective uncapping strategy (Kim, Rivera et al. 2001; Lin, Smith et al. 2004) and a synergistic effect was seen when this was combined with expression of hairpin short-interfering RNA against hTERC RNA. (Li, Rosenberg et al. 2004)

Current estimates for new drug development approximate 15 years in trials and a cost of around $500 million. (NCI 2002) If a drug with fully understood pharmacokinetic and pharmacodynamic properties is effective in the context of targeted therapy then clearly there will be a substantial reduction in time to clinical trials. Thus while targeted therapy addresses the very fundamental specific differences of the cancer cell at the genomic level, agents to produce effect need not be entirely novel and complex in nature.

8.2.4. Radiosensitisation of malignant cells.

Ionising radiation has proved to be an effective anticancer therapy in the context of SCCHN. Interestingly, the mode of radiation induced cell death and the true basis of the increased radiosensitivity of the malignant versus the normal keratinocyte remains obscure. There is substantial toxicity with standard therapies and many patients require hospital admission for supportive therapy during the course of standard schedules. There is also a mortality rate in patients undergoing radiotherapy. Radiation sensitivity also varies both between and within tumour types.

Both of these factors have driven the search for radiosensitising agents which might enhance therapeutic effectiveness and limit toxicity to normal tissues. Over time two main classes of drugs have emerged; halogenated pyrimidines and hypoxic cell sensitisers. These are now discussed in turn.

Halogenated pyrimidines are incorporated into the growing DNA strand in place of the normal thymidine precursor. These molecules have a halogen (chlorine, bromine or iodine) incorporated instead of the methyl group. The theoretical basis upon which experiments with these agents was based is that these agents are incorporated to a greater extent in to the DNA in malignant tissue due to faster cell cycle time in the tumour population versus normal tissue. The DNA strand is then thought to be more susceptible to DSBs induced by ionising radiation and hence radiosensitise the tumour. Importantly, these substances were found to be effective only if they were incorporated into DNA over successive generations, thus increasing the amount of analogue included and hence maximally weakening the DNA strand. This contrasts with the early effect of AZT shown in our experiments. The effectiveness of such drugs (bromodeoxyuridine and iododeoxyuridine) was shown both *in vitro* and *in vivo* and shown to be similar for both agents with x-rays. (Mitchell, Morstyn et al. 1984) Initial trials in head and neck cancer patients proved however to have unacceptable levels of normal tissue damage. Some reports of use of these agents in other cancers have proved more promising (e.g. high grade gliomas which comprise rapidly growing tumour tissue with very slowly growing, or non-growing surrounding tissue)

(Sullivan, Herscher et al. 1994) More recently, newer agents in this class of molecule have been investigated. Gemcitabine (2'2'-difluoro-2'-deoxycytidine) is another pyrimidine analogue which has shown promise in a number of preclinical models including head and neck cancer. (Shewach and Lawrence 1996; Mason, Milas et al. 1999; Robinson and Shewach 2001; Sangar, Cowan et al. 2004) (Fields, Eisbruch et al. 2000) Importantly, incorporation of the analogue into cellular DNA seems to depend on a high growth fraction and labelling index. (Begg, Haustermans et al. 1999) It may be therefore that this approach would be more helpful if targeted to such tumours.

Hypoxic cell sensitisation has been attempted using both hyperbaric oxygen and hypoxic radiosensitising agents. Increasing oxygen tension within hypoxic tumour tissue facilitates the production of free radicals which produce indirect DNA damage (estimated to comprise approximately 70% of total X-Ray and γ-Ray ionising radiation induced DNA damage).

The possible benefit of hyperbaric oxygen therapy in improving tumour oxygenation and hence radiation cell kill was first investigated in the middle of the last century by Churchill-Davidson. (Churchill-Davidson, Sanger et al. 1955) The clinical trials which followed involved small numbers of patients and generally were hampered by the logistics of use of the hyperbaric chamber. As a consequence, unconventional fractionation regimes tended to be employed (fewer larger fractions). Overall, hyperbaric radiotherapy was shown to be superior to radiotherapy in air, producing a 6.6% improvement in local control. (Overgaard 1995) The use of this therapy has however now diminished markedly with some authors suggesting increased morbidity as a factor in this. (Overgaard 1995)

Enhancing oxygen delivery to the tissues generally is facilitated by an appropriate circulating haemoglobin (Hb) concentration. This has been shown to be of significance in the context of SCCHN (Overgaard, Hansen et al. 1989), where local control is favoured by higher Hb levels. This data is not straightforward to interpret as patients with aggressive, rapidly advancing disease which responds poorly to any treatment modality are most likely

to also be anaemic as part of their systemic disease process. (Dische 1991) Further, the immunosuppressive effect of red cell transfusion producing increased recurrence rates must be considered. Trials using erythropoietin to enhance haemoglobin with radiotherapy in SCCHN patients have proved disappointing. (Henke, Laszig et al. 2003)

Hypoxic cell radiosensitisers are chemical agents which are aimed to mimic oxygen in sensitising tumour cells. Importantly, such agents require to be highly lipid and water soluble and not be metabolised rapidly by the cell. These features will therefore allow continued diffusion through cancer tissue to reach those cells most distant from the capillary bed, which are normally relatively radioprotected by their hypoxic state. The nitroimidazoles are a group of related compounds which have been extensively investigated in this regard. The first to be analysed was metronidazole, an antibiotic agent active against anaerobic organisms. In order to effectively radiosensitise, ten-fold higher doses were required to be used and induced severe nausea and vomiting (Urtasun, Band et al. 1976) Misonidazole was used widely in trials, but doses used clinically were limited by peripheral neuropathy which extended to central nervous system involvement if therapy was not stopped. (Saunders, Dische et al. 1978) Etanidazole, pimonidazole and nimorazole were the next generation of nitroimidazoles to be examined. Etanidazole showed no advantage in two large head and neck trials carried out, one in Europe and one in the USA. (Chassagne, Charreau et al. 1992; Lee, Cosmatos et al. 1995) Subsequent studies in early disease have confirmed this outcome. (Eschwege, Sancho-Garnier et al. 1997) Pimonidazole in fact showed adverse outcome in the treatment arm of one study, thought to be attributable to the vasoconstrictive effect of the compound actually exacerbating radioresistance by further increasing hypoxia within tumour tissue. (Dische, Bennett et al. 1989) Significantly, nimorazole was shown to confer a therapeutic advantage in a Danish study. (Overgaard, Sand Hansen et al. 1991) This effect was only seen in subgroup analysis, and was restricted to supraglottic and pharyngeal tumours only. A further finding from this research was that Hb level also made a significant difference in both control and

nimorazole treated groups. (Overgaard, Sand Hansen et al. 1991) A further randomised, double-blind trial from the same group has confirmed this finding, focusing in this study on supraglottic and pharyngeal SCCHN only. Here the nimorazole treated group had 49% local control versus 33% in the control group. (Overgaard, Hansen et al. 1998) In summary, the nitroimidazoles have been shown to produce an effect which is only modest and only in certain cancer subtypes.

8.2.5. Targeted radiosensitisation.

While the radiosensitisation principal aims to be selective for tumour cells, this specificity of action will be most effective if targeted at pathways and molecules specific to the tumour cell. Some recent preclinical work in radioresistant tumour models has targeted tumour angiogenesis. In both glioblastoma multiforme and malignant melanoma tumours, anti-VEGF2 antibodies have been shown to modify tumour vasculature and reverse radioresistance. (Geng, Donnelly et al. 2001) This is an interesting outcome, since reduced vascularity and resulting cellular hypoxia could be expected to reduce radiosensitivity, but in fact the opposite was observed.

8.2.6. Telomerase inhibition and radiosensitisation;

In the telomerase inhibition experiments in this work reduction of telomerase activity had three main effects in the malignant cells studied. Cloning efficiency was reduced in keeping with the findings of other authors (Blasco 2002). This has been shown to be a far more marked effect in cancer cells versus normal cells. (Murakami, Nagai et al. 1999) Secondly telomere function was reduced and this was accompanied by a third effect; radiosensitisation. The focus of this work was to assess the effect of changing telomere function on radiation survival. The means by which this observed sensitisation is occurring is intriguing and central to considerations of how any novel agent might act and therefore be discovered.

Work by the Weinberg group described the post senescent telomere as having an eroded 3'-single stranded end. (Stewart, Ben-Porath et al. 2003) It is possible and has been

suggested that in this circumstance the shortened telomere relies on the telomerase enzyme for capping, in a fashion distinct from the maintenance of repeats. (Blackburn 2001) Certainly, AZT is rapidly effective in producing telomere dysfunction in BICR 6, suggesting an effect distinct from telomere erosion accompanying cell division which would take some time. This rapid onset telomere dysfunction may be simply producing failure to bind signalling or assessing gene products which are then more freely available to signal cell damage and induce cell death. Perhaps the most appealing answer currently offered by experimental evidence for the radiosensitising effect of impaired telomere function with telomerase inhibition is that described by Latre *et al* where uncapping of the short telomere simply allows inappropriate chromosomal fusion and hence loss of cellular viability.(Latre, Tusell et al. 2003)

A different and perhaps contributing explanation might be that the induced telomere dysfunction simply causes cell cycle arrest in mitosis by anaphase bridging. This is a particularly radiosensitive cell state regardless of other parameters. This type of cell cycle synchronisation effect has been shown to occur using docetaxel in colon cancer cells where a G2/M arrest was noted. (Dunne, Mothersill et al. 2004) Intriguingly taxanes like docetaxel and paclitaxel have been used experimentally and shown to target telomeres for degradation and so could conceivably be radiosensitising, at least in part, by induction of telomere dysfunction. (Mo, Gan et al. 2003) The addition of docetaxel to standard chemoradiation for SCCHN has also resulted in increased tumour response rates in clinical trials currently underway. (EORTC 2004) Assessment of telomere function in cancer cells in this context could certainly yield valuable information.

Studies using BIBR 1532 have shown a progressive telomere shortening resulting ultimately in proliferation arrest with hallmarks of senescence (Damm, Hemmann et al. 2001) and cell death. (El-Daly, Kull et al. 2004) Notably in leukaemia studies, telomere length was significant in predicting time to cell death which was not dependent on telomerase catalytic activity levels. Work in telomerase knockout mice showed that

telomere shortening after successive generations of progeny produced radiosensitisation in the normal mouse cell. (Goytisolo, Samper et al. 2000; Wong, Chang et al. 2000) Assessing the effect of BIBR1532 on radiation survival might therefore help in dissecting whether induced telomere dysfunction *per se* or telomere length reduction is the more significant change. Again, however, although the murine model may provide some insight and give direction for future research, any effect in human cancer could only be evaluated in human cancer cell systems and ultimately in clinical trials.

BICR6 is a SCCHN cell line with high levels of telomerase expression. Although this was successfully inhibited in our experiments, we were also able to demonstrate that with tumour progression to recurrence both in cell lines and *in vivo* specimens telomere function improved. This implies further upregulation of telomerase with clonal evolution in the cancer population. Clearly the inhibition of the telomerase enzyme complex will be most effectively achieved clinically where endogenous levels of expression are lower. Thus we would predict that telomerase inhibitor chemotherapy would be most effective in early disease, or even as a chemopreventive agent in the context of management of dysplastic epithelium. This may have relevance in clinical research with any novel antitelomerase therapeutic in early studies in patients with late stage disease, where a reduced effectiveness might be expected.

The finding that SF4 correlated in a non-linear fashion with telomere function further corroborates that radioresponsiveness and telomere function may be linked. If borne out by further studies this could become a component of individualisation of therapy for cancer patients in the future, when telomerase inhibitor agents become available clinically

8.4 Further Research plans

In taking these research findings further, establishing the impact of AZT in other SCCHN lines would be a priority. We selected BICR6 as a cell line with typical p16, p53 and pRb status, with high endogenous telomerase expression and low level anaphase bridging.

Assessing the telomerase inhibition, telomere function and radiation survival effects on other SCCHN lines would further enhance our confidence in this therapeutic approach.

Novel agents like BIBR1532 which have been shown to inhibit telomerase effectively would also be of relevance in experiments with malignant keratinocytes and might enhance our understanding of the radiosensitisation mechanism.

Experiments in nude mice with subcutaneous SCCHN xenografts could provide comparison of control animals with those fed AZT in an *in vivo* animal model. Tumour responsiveness would be assessed and histologic assessment of ABI and TIF assays would provide data regarding induced telomere dysfunction. The pharmacologic properties of AZT are already well established and so should the above research provide further data supporting the telomerase inhibition radiosensitisation hypothesis then progress to clinical trials could be rapid.

Current accepted management of SCCHN involves surgery and radiotherapy, implemented according to clinical criteria. All patients who have unfavourable clinical parameters after primary surgery will progress to external beam teletherapy. This affords us an excellent opportunity to assess the impact of a telomerase inhibitor agent in this population. A randomised, doubleblinded prospective trial of telomerase inhibitor (perhaps AZT) versus placebo could run within the existing treatment infrastructure. This approach would be immediately selecting out patients in the poorest prognosis post surgery group, who are most likely to develop recurrent disease. This means that any reduction in recurrence would be best demonstrated in these patients and that correspondingly the best clinical impact would be seen here. Further, most patients who will have recurrence would be expected to have this relatively soon in disease course (approximately 85% within 2 years of primary therapy) and so initial survival data differences would be accumulated relatively quickly. Adopting this strategy would also mean that the telomerase inhibitor agent would be administered perhaps only during the six weeks of radiotherapy. Therefore should an

agent like AZT be used drug toxicity would be expected to be lower than in patients on long term therapy (e.g. in HIV disease).

8.5 Conclusion.

We are now in the dawn of the era of truly targeted cancer therapy based on our understanding of novel molecular targets. In sight of these targets, we now require the "magic bullets" first described by Ehrlich in the context of sepsis. Our advanced understanding means that we realise that rather than bullets, we require missiles with both a delivery system and the anticancer payload. Novel agents like BIBR1532 show early promise and will surely be followed by others. Importantly, scrutiny of mechanism may allow the use of agents already employed in other therapeutic contexts and AZT is a good example of this.

The telomere and its maintenance by telomerase are a good example of an issue in human medicine which has been described some time ago and has its full therapeutic potential realised many years after first description. In the 40 years since Barbara McClintock and Leonard Hayflick first published on the subject there has been a lull followed by a build to a worldwide momentum behind this therapeutic approach. (Mc 1951; Hayflick 1961) There will surely be developments in this field in the near future. In the face of a cancer which is relatively easy to diagnose and monitor, SCCHN remains stubbornly resistent in terms of prognosis. The need for new targeted therapy to improve outcome with minimal toxicity in this disease is clear. The West of Scotland is a world-wide disease focus for SCCHN and so the need here is great. The corollary of this is that further laboratory investigation followed by well designed and run clinical trials will be facilitated by the Scottish population disease burden and positive outcomes can have the maximum benefit in these patients. Telomerase represents one of the most promising cancer molecular targets under study today. Further lab investigation must be followed promptly by

translational research in order to maximise its potential for our cancer patients in the shortest possible timeframe.

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Additional Material

2nd European Conference on Head and Neck Cancer; Lille 2003

Golden Award for Best Research Poster; Telomerase Inhibition with AZT radiosensitises head and neck cancer cells

The search for new and more specific cancer therapies continues. Due to the end replication problem, telomeres at the chromosome ends shorten with each replication of the cell. The immortalisation of human cancer cells requires maintenance of these telomeres through many rounds of replication. In more than 90% of all human tumours this is achieved by the upregulation of the telomerase enzyme; a RNA dependent reverse transcriptase. In epithelial cancers, including squamous carcinoma of the head and neck, the percentage of tumours utilising this mechanism may be even higher. This distinction between normal tissue and malignant cells represents an attractive target for new, more specific anticancer therapies.

The telomere has many functions and many associated proteins. These include proteins involved in repair of DNA double strand breaks (DSB). The DSB is the lethal cellular injury produced by therapeutic ionising radiation. Telomerase inhibition, producing telomere shortening and/ or dysfunction may therefore affect the radiosensitivity of cancer cells

Here we describe experiments where we have increased telomerase levels in cancer cell lines derived from *in vivo* human squamous cancers by stable transfection of the hTERT gene. In cells with low endogenous telomerase this intervention produced radioprotection. We further describe experimental data from work using the reverse transcriptase inhibitor AZT (3'-azido-deoxythymidine) to inhibit the telomerase enzyme in human oral squamous carcinoma cells. Levels of telomerase suppression achieved (assayed using the TRAP PCR method) and corresponding SF2 (surviving fraction after two Grays of radiation) are discussed.

6th International Conference on Head and Neck Cancer, Washington DC. August 2004

Abstracts shortlisted for the Best Basic Science Paper by a Resident and presented August 2004

6thIntHN_2004__130000

p53 codon 72 polymorphism, telomere function and radiation survival in SCCHN lines

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Introduction
p53 is the most widely mutated tumour suppressor gene in human cancer. An important polymorphism occurs in exon four at codon 72 which may be either an arginine (Arg 72) or proline (Pro 72) residue. A gradient exists within the human population, with the arginine variant becoming more prevalent with distance from the equator. This is thought to be linked to the ability of the Arg 72 variant to induce apoptosis in response UV induced DNA damage. In human cancer the arginine form of the protein is selected for and may in fact, when mutated, act as an oncogene(s), by interfering with the tumour suppressive effects of both residual wild type p53 and other p53 family members (p63 and p73). Recent evidence suggests that cancers with mutated p53 Arg 72 have reduced response rates to chemotherapy, seen as reduced rates of apoptotic cell death. We aimed to assess any effect of Arg72 mutated p53 on radioresistance.
We have shown previously that manipulation of the telomerase enzyme, which is functionally specific to cancer cells, results in altered radiation survival in SCCHN. The anaphase bridge index (ABI) provides a functional assessment of the telomeres, which depend on telomerase for maintenance in cancer cells. In this study we analysed all SCCHN lines for telomere function in order to assess for correlation with radiation survival.
Methods
We examined the codon 72 status of a group of 16 in vivo derived SCCHN cell lines by sequencing p53 exon four in genomic DNA harvested from growing cultures. The influence of this status on radiation survival was assessed after 2Gy and 4Gy of radiation delivered in an Alcyon II 60Co radiation source. Cells were replated and cultured and cloning efficiency was calculated. All experiments were repeated in triplicate.
Further information on the correlation of telomere function with radiation survival was investigated by assessment of anaphase bridge indices (ABI) for each cell line.
Results
An excess of Arg 72 polymorphisms was found in the mutated p53 in this series. No significant differences in radiation survival between Arg72 and Pro72 p53 mutations could be demonstrated. Telomere function analysed together with radiation survival assess for correlation showed no link at 2Gy. However the 4Gy survival fraction for SCCHN was shown to correlate with the level of telomere dysfunction ($p<0.001$, R square =0.82). This non-linear correlation is given by the model ABI = 5.107/SF4.
Conclusions

The Arg72 polymorphism is more prevalent in SCCHN derived from Scottish patients, in keeping with other latitude based studies. There was no demonstrable radioresistant effect of gain of function p53 mutation for either polymorphism. We have shown a direct correlation between telomere function in SCCHN and radiation survival for these tumours. ABI may therefore be a useful tool firstly to predict the effectiveness of radiotherapy for any given tumour and also to predict any enhance radiotherapy effect produced by a telomerase inhibitor agent.

6thIntHN_2004__131000

Telomerase Inhibition with AZT radiosensitises head and neck cancer cells

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Introduction.

Telomerase is a new, potentially highly selective cancer therapeutic target. Telomeres at the chromosome ends shorten with cell replication. The immortalisation of human cancer cells requires maintenance of these telomeres through many rounds of replication. In more than 90% of all human tumours this is achieved by the upregulation of the telomerase enzyme; a RNA dependent reverse transcriptase. In epithelial cancers, including squamous carcinoma of the head and neck (SCCHN), the percentage of tumours utilising this mechanism may be even higher. This distinction between normal and malignant cells represents an attractive target for new, more specific anticancer therapies.
The telomere has many functions and associated proteins including those involved in repair of DNA double strand breaks (DSB). The DSB is the lethal cellular injury produced by ionising radiation.

To investigate whether telomerase inhibition, producing telomere shortening and/ or dysfunction affects the radiosensitivity of cancer cells we increased telomerase levels in cancer cell lines derived from in vivo human squamous cancers by stable transfection of the hTERT gene and assessed radiation survival. We further describe experimental data from work using the reverse transcriptase inhibitor AZT (3'-azido-deoxythymidine) to inhibit the telomerase enzyme in human oral squamous carcinoma cells.

Methods.

SCCHN cells were stably transfected with hTERT using phoenixA retrovirus carrying the pBabepuro plasmid with an hTERT construct inserted. Thus large increases in cellular level telomerase expression were produced. Cells derived from tumours with low endogenous telomerase expression (BICR 7) were compared with those from tumours with high telomerase expression (BICR 6). Both empty vector treated cells and hTERT expressing cells were then irradiated. Survival curves were calculated by replating and culturing colonies and comparing irradiated with non-irradiated cells. As controls we assessed non malignant human cells (HFFs) and also the rare ALT cell line GM847. These latter cells are immortal fibroblasts which do not use telomerase. These controls underwent the same genetic manipulations.

The effect of telomerase inhibition was then assayed by culturing wild type tumour cells with high telomerase expression (BICR 6) for seven days in medium containing the small molecule reverse transcriptase inhibitor 3'azido-, 3'deoxy-

thymidine (AZT) in a previously calculated dose range. The PCR based telomere repeat amplification protocol (TRAP) assay was used to measure telomerase inhibition. These were irradiated with 2Gy of ionising radiation, recultured and survivals curves calculated.

As controls we used the same tumour cells transfected with hTERT in which AZT would be predicted not to be capable of producing telomerase inhibition and also GM847 ALT cells.

Results

In cells with low endogenous telomerase increasing expression produced statistically significant increases in radiation survival. Where telomerase inhibition was achieved with AZT (assayed using the TRAP PCR method) corresponding SF2 (surviving fraction after two Grays of radiation) was reduced.

Conclusions

Manipulating telomerase expression cell lines affects radiation survival specifically in in vivo derived SCCHN. AZT can effectively inhibit telomerase in these cells.

These data provide proof of concept for the therapeutic potential of telomerase inhibition as a targeted strategy in SCCHN management.

Joint British and American Association of Oral and Maxillofacial Surgeons Conference, Killarney, Ireland, 2002

Abstract accepted for presentation

Telomerase Inhibition and the Radiosensitisation of Head and Neck Cancer

McCaul J A, Parkinson, E. K.

Chromosome integrity depends on the function of telomeres at the chromosome ends. These repeats of DNA (TTAGGG)n and associated proteins shorten with cell division until the cell cannot replicate. The germ line avoids this with telomerase, which maintains the telomere. Telomerase is not active in normal cells, but tumours must maintain telomere length and function and do this in most cases by reactivating telomerase. Telomerase inhibition shortens telomeres and causes apoptosis. Furthermore, as tumour cells have short telomeres, they have increased susceptibility over normal cells. Telomerase is therefore an attractive, highly selective target. The telomere also functions in DNA double strand break repair and dysfunction provokes radio-sensitivity in mice. Thus inhibiting telomerase may enhance the effectiveness of radiotherapy.

Two cancer cell lines derived from oral cancers with functional (BICR6) and dysfunctional (BICR7) telomeres were selected for study. Each had the hTERT gene inserted to increase telomerase expression. In BICR6 where high levels of telomerase are expressed constitutively there is little evidence of telomere dysfunction. Overexpression of telomerase had no survival effect. However BICR7 expresses low levels of telomerase and displays telomere dysfunction. Here target cells became radioresistant .

Experiments radiosensitising head and neck carcinoma by inhibiting telomerase are in progress.

British Association of Oral and Maxillofacial Surgeons Conference, Glasgow, 2003

Abstract accepted for presentation.

Due to the end replication problem, telomeres at the chromosome ends shorten with each replication of the cell. Immortalisation of human cancer cells requires maintenance of telomeres through many rounds of replication. In more than 90% of all human tumours this is achieved by upregulation of telomerase; a RNA dependent reverse transcriptase. In epithelial cancers, including squamous carcinoma of the head and neck, the percentage of tumours utilising this mechanism may be even higher. This distinction between normal tissue and malignant cells represents an attractive target for new, more specific anticancer therapies.

The telomere has many functions and associated proteins. These include proteins involved in repair of DNA double strand breaks (DSB). The DSB is the lethal cellular injury produced by ionising radiation. Telomerase inhibition, producing telomere shortening and/ or dysfunction may therefore radiosensitise cancer cells

We describe experiments where we have increased telomerase levels in cancer cell lines derived from *in vivo* human squamous cancers by stable transfection of the hTERT gene. In cells with low endogenous telomerase this intervention produced radioprotection. We further describe experimental data where the reverse transcriptase inhibitor AZT (3'-azido-deoxythymidine) inhibited the telomerase enzyme in SCCHN cells. Levels of telomerase suppression achieved (assayed using the TRAP PCR method) and corresponding SF2 (surviving fraction after two Grays of radiation) are discussed.

Scottish Oral and Maxillofacial Surgery Meeting Abstracts

Prize for Best Research Paper; Crosshouse, Kilmarnock 2001. Telomerase inhibition and the future management of SCCHN.

The telomerase enzyme complex represents a specific target for future cancer therapy. Correlative evidence suggests that the short dysfunctional telomere radiosensitises the cell. We investigated the role of telomerase in radiosensitivity in two SCCHN cell lines; BICR6 and BICR7. Our data show that in the presence of poor telomere function, ectopic expression of telomerase radioprotects the cancer cell. This provides evidence for the role of the telomerase enzyme complex in radiation survival for SCCHN tumours.

Prize for Best Research Paper; Perth 2003; P53 codon 72 polymorphism and radiation survival in SCCHN lines.

P53 is the most widely mutated tumour suppressor gene in human cancer. An important polymorphism occurs in exon four at codon 72 which may be either arginine or proline. In human cancer the arginine form of the protein is selected for and may in fact, when mutated, act as an oncogene.
This study examines the codon 72 status of a group of *in vivo* derived SCCHN cell lines and assesses the influence of this status on radiation survival.

T.C.White Prize Lecture. The Royal College of Physicians and Surgeons of Glasgow. Awarded February 2003

Abstract for T. C. White Prize Lecture

Telomerase Inhibition in Squamous Carcinoma of the Head and Neck

The overall five year survival rates for squamous Cancer of the Head and Neck have remained unchanged for 30 years. An exciting new therapeutic possibility is inhibition of the enzyme telomerase. This project examines the effectiveness of telomerase inhibition on tumour growth and in particular targets the radiosensitivity of this tumour type when telomerase is inhibited and the chromosome telomeres shorten.

Telomeres are structures forming caps at the ends of eukaryotic chromosomes which maintain their integrity. These are comprised of repeats of DNA (TTAGGG)n and associated proteins. Because of the end replication problem, the telomere is known to shorten with every cell division. Eventually, the telomere is so short that the cell can no longer replicate. In germ line cells this problem is avoided by the enzyme telomerase which maintains the telomere repeats and therefore cell division can continue. Telomerase is not active in normal somatic cells, which can only carry out a finite number of cell divisions. Human tumours, including squamous cell carcinoma of the head and neck

(SCC-HN), need to maintain telomere length and function. They do this in most cases (85-90%) by reactivating telomerase. Inhibiting telomerase has been shown to lead to apoptosis. Furthermore, as most tumour cells have very short telomeres, they are more likely to succumb to telomerase inhibition than normal cells. Telomerase is therefore an attractive, potential, highly selective anti-cancer target. The telomere also has a role in the repair of DNA double strand breaks and telomere dysfunction provokes radio-sensitivity in mice. Thus inhibiting telomerase may produce enhanced effectiveness of radiotherapy for cancer by selectively radiosensitising telomerase positive cancer cells.

Cells derived from human oral squamous carcinomas are being used to investigate this hypothesis. The existence of dicentric chromosomes and/ or anaphase bridges are a good correlate of telomere dysfunction and two cell lines with functional (BICR6) and dysfunctional (BICR7) telomeres were selected for study. In BICR6, where the tumour constitutively expresses high levels of telomerase already and shows little evidence of telomere dysfunction there was no effect on radiosensitivity. However BICR7 expresses low levels of telomerase and displays telomere dysfunction and in this case, the overexpression of telomerase rendered the target cells radioresistant.

Experiments designed to radiosensitise head and neck carcinoma by inhibiting telomerase are in progress. Additionally, tumours specimens are being collected from patients undergoing surgery for oral cancer in order to fully assess the telomere lengths and telomerase levels in these cancers in the Scottish population.

SEHD/CSO online Research report 2004

Scottish Executive Health Department Chief Scientist Office

FOCUS ON RESEARCH

TELOMERASE, TELOMERE LENGTHS AND RADIOSENSITISATION WITH TELOMERASE INHIBITION IN HEAD AND NECK CANCER

Researchers
Dr J A McCaul, Dr E K Parkinson.

Aim
1. To determine whether manipulation of the telomerase enzyme in squamous cell carcinoma of the head and neck (SCCHN) affects radiosensitivity of this cancer type.
2. To assess the effect of different forms of the mutated p53 protein on radioresponsiveness of SCCHN.

Project Outline/Methodology
This was a lab-based investigation involving the culture of SCCHN derived cells in a cell culture system which reproduces closely the conditions seen in patients. The telomerase enzyme is a very new target in cancer and is a protein which maintains the chromosome endcaps. These are called the telomeres. Using cell lines with defined levels of telomerase protein and known levels of telomere dysfunction, we were able to manipulate telomerase by both transferring human genes into cells and by using small molcule drugs. This allowed us to assess the response to ionising radiation delivered to the cancer cells in a machine used for cancer treatment and compare radiation survival. As controls we assessed cancer cells without the added genes, normal cells, and cells immortalised by a rare mechanism termed ALT. These last cells are immortal but do not use the telomerase enzyme. We aimed to assess the usefulness of staining with antibodies in estimating telomerase levels in SCCHN. We investigated the function of the telomeres in primary and recurrent SCCHN, to provide evidence for upregulation of telomerase with tumour progression as this might influence the success of treatment in late stage cancer. Further work included the sequencing of mutated p53 in a number of SCCHN lines

Key Results
We demonstrated clear radioprotection produced by increasing telomerase levels in cancer cells with low levels of this protein ($P<0.01$). This effect was specific to cancer cells using telomerase to maintain their immortality, being absent in normal cells and in ALT cells. We then were able to demonstrate that AZT, an antiretroviral drug widely used to treat HIV disease could inhibit telomerase, produce dysfunction of the telomeres and enhance the effectiveness of radiation in producing cell death ($p<0.01$). Our investigation of recurrent tumours showed that with tumour progression telomere function improves. SCCHN lines derived from recurrent cancer were not significantly more radioresistant.

Conclusions
Telomerase inhibition can be used to enhance the effectiveness of radiotherapy for SCCHN. With more advanced disease, telomerase levels are likely to be higher and so this potential therapeutic benefit may be more difficult to achieve.

What does this study add to the field?
Previous work has shown a correlation between telomere length and radioresistance in other cancers. In mice, inhibition of telomerase and the resulting shortened telomeres over successive generations have been shown to become radiosensitive. We have provided the first evidence in eukaryotic malignant cells for the radiosensitising of human cancer cells by inhibiting telomerase.

Implications for Practice or Policy
Inhibition of telomerase in patients with head and neck squamous cancer may provide useful therapeutic advantage when combined with radiotherapy. This effect can be produced with AZT. This new strategy fits well with the current NHS treatment infrastructure already in place for these patients. This approach is also most likely to be effective in early stage disease.

Where to next?
Clinical trials using this strategy will provide insight into how effective telomerase inhibition with radiation will be in enhancing survival in SCCHN. Determining the effect in other tumour types will allow the likely effect of this strategy in other cancers to be assessed.

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McCaul J A et al. Lancet Oncology; 2002. 3(5): p. 280-8.

Telomerase inhibition and the future management of head-and-neck cancer

James A McCaul, Katrina E Gordon, Louise J Clark, and E Kenneth Parkinson

Telomeres are tandem repeats of DNA associated with specific proteins. These structures cap eukaryotic chromosomes and maintain the integrity of the chromosome ends. In the germline, telomeres are maintained by the enzyme telomerase, but in normal somatic cells the enzyme's activity is low or undetectable. Human tumours, including squamous-cell carcinoma of the head and neck (SCCHN), need telomerase to maintain telomere function; inhibition of the enzyme can lead to apoptosis. Furthermore, because most tumour cells have very short telomeres, they are more likely to succumb to telomerase inhibition than normal cells. Telomerase is therefore a potential selective anticancer target. The telomere is also involved in the repair of DNA double strand breaks, and telomere dysfunction provokes radiosensitivity. In this review we consider whether manipulation of telomere function may selectively sensitise SCCHN to radiotherapy and discuss the possible pitfalls. We also assess how some conventional treatments may affect the subsequent use of telomerase inhibitors.

Lancet Oncol 2002; 3: 280–88

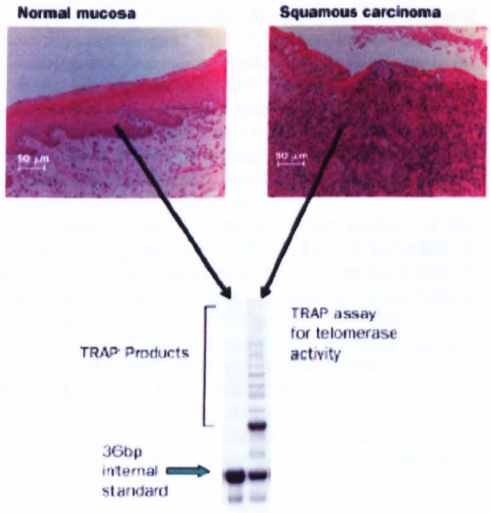

Figure 1. Telomerase activity in SCCHN. The telomere-repeat amplification protocol (TRAP), a PCR-based technique, assesses telomerase activity. Normal epithelium (left) produces no reaction products. By contrast, tumour cells (right) show reaction products extending along the gel, indicating telomerase activity.

The overall 5-year survival for squamous-cell carcinoma of the head and neck (SCCHN) has not changed during the past 20 years. Despite advances in surgical management and radiation oncology, the 5-year survival proportion of 45–50% remains static.[1] A promising target for new approaches to cancer therapy has recently emerged, in the form of the enzyme telomerase. Possible strategies directed against telomerase have been reviewed many times.[2-4] Here we focus on the recent developments in the field, in the context of SCCHN, because about 90% of these cancers show detectable telomerase activity (figure 1).[5] In particular, we discuss the possible use of telomerase inhibitors to selectively radiosensitise SCCHN cells and the possible limitations of this approach. We also review the use of angiogenesis inhibitors in cancer and the proposal[6] that this new class of drugs might be used to complement telomerase inhibitors.

Telomeres and the end-replication problem

Telomeres consist of tandem repeats of the DNA sequence TTAGGG and associated proteins; they function to cap the ends of human chromosomes. Capping prevents chromosomes from fusing together and stops their ends being misinterpreted as DNA double-strand breaks.[7] Telomeres also act as repositories for DNA repair enzymes[8] and can silence genes close to them.[9]

Telomeres, lying at the ends of the linear DNA molecules, are subject to the end-replication problem,[10] whereby the lagging strand of the molecule is incompletely replicated owing to removal of the last RNA primer (figure 2).[11] Telomere sequences may also be lost by exonuclease digestion[12] and oxidative damage;[13] unless mechanisms are in place to counter this loss, the telomeres shrink with each cell division. In the germline, telomere loss is countered by the presence of telomerase,[14] which balances the loss by the direct addition of TTAGGG repeats to the chromosome

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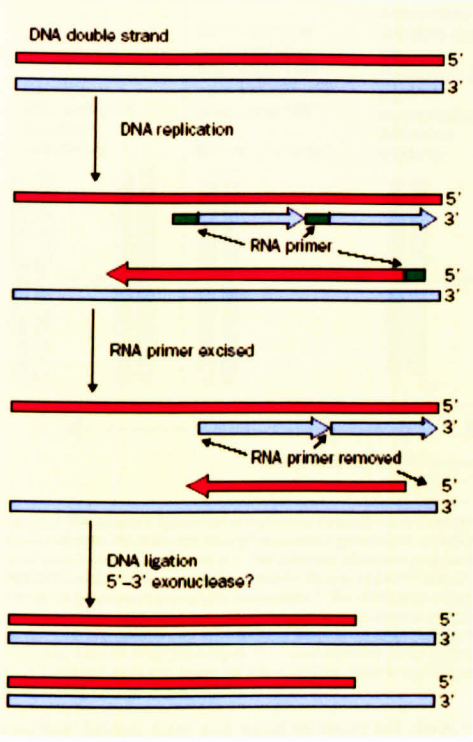

Figure 2: The end replication problem and telomeric attrition. The figure (modified from reference[1]) illustrates the end replication problem and how it contributes to telomeric attrition in the absence of telomerase. Removal of the RNA primer at the 5' end of the newly replicated DNA strand leads to a gap that cannot be filled by DNA polymerase. Further digestion of this strand by a putative 5'-3'-exonuclease is proposed to increase the size of the gap, so that the double-stranded DNA molecules have a 3' single-strand overhang at their telomeres. Repetition of these processes results in progressive attrition of 5' ends (red strand), thus leading to a gradual shortening of the telomeres after many cell divisions in the absence of telomerase.

end.[15] Telomerase consists of at least two essential components, the RNA template (TERC)[16] and the catalytic subunit (TERT).[17] There is now evidence that the final length of the telomere is under very sophisticated control. In particular, in the germline, telomere length can be reset when animals are cloned from senescent somatic cells, but the mechanisms have yet to be defined.[18]

The telomere hypothesis of replicative senescence

In the latter stages of human development, telomerase activity is completely repressed in most somatic cells[19] and downregulated in haemopoietic stem cells,[20] which means that telomere length is not adequately maintained throughout life. The ensuing telomeric attrition has been proposed as the basis of a cellular replicometer, which can both count and remember the number of cell divisions completed by a telomerase-deficient cell.[21] This idea is supported by the observations that telomeres, as assessed by the size of telomere repeat fragment lengths after restriction-enzyme digestion of the DNA, shorten both in vivo and in vitro;[20,22] that the replicative lifespan of human fibroblasts can be predicted on the basis of starting telomere length;[23] and that telomeric attrition both in vivo and in vitro depends on cell division.[24] Most importantly, the correction of telomere dysfunction by the expression of the telomerase catalytic subunit, TERT, can reconstitute telomerase activity[24] and extend the lifespan of human fibroblasts[25] without causing karyotypic changes or features of transformation.[26] Since the cloning of the first essential telomerase component, TERC,[16] the creation of strains of telomerase-deficient mice (TERC-/-) that show telomeric attrition has become possible.[27] Even though mice have much longer telomeres than human beings,[28] breeding of TERC-/- mice through four to six generations leads to compromised proliferation and loss of viability of the germline and lymphocytes, sterility, poor wound healing, and many features of human ageing.[27-29]

In human beings, a mutation in the product of the dyskeratosis congenita gene, dyskerin, leads to instability of the *TERC* transcript and a reduction in telomerase activity.[30] Fibroblasts from these individuals cannot be immortalised by the expression of the *TERT* gene because there is too little of the subunit to generate the required enzyme activity. Dyskeratosis congenita is characterised by early-onset anaemia and bone-marrow failure, resembling the disorders seen in telomerase-null mice at later generations. Therefore, collectively, these studies support the notion that telomeric attrition prevents indefinite clonal proliferation in vivo.

Human cellular mortality and the role of telomeres

Most somatic human cells, including the keratinocytes of squamous epithelium, are telomerase deficient and mortal in vitro. In cultured epithelial cells, replicative senescence is thought to be controlled first by the accumulation of $p16^{INK4A}$ (mortality mechanism 0 or M0). M0 is not directed by telomere length,[31] but it seems to depend on the rate of oxidative damage, the cell type, and the culture environment.[32] Putative telomere-independent mechanisms of replicative ageing reflect inadequate growth conditions.[32] However, we emphasise that there is no direct evidence that oxidative damage drives the accumulation of $p16^{INK4A}$, or M0, and the role of M0 as a checkpoint to the development of cancer in vivo has not been firmly established. After further divisions and a period of telomere loss, these cells then encounter mortality mechanism 1 (M1; figure 3).[33] M0 and M1 are biologically indistinguishable;[34] both are cell-cycle arrest states and are referred to as replicative senescence. After disruption of the cell-cycle control pathways (and M0 and M1) by, for example, DNA tumour viruses, the telomeres shorten still further, until one or more becomes dysfunctional. At this time, as a consequence of telomeric fusions, many dicentric chromosomes and

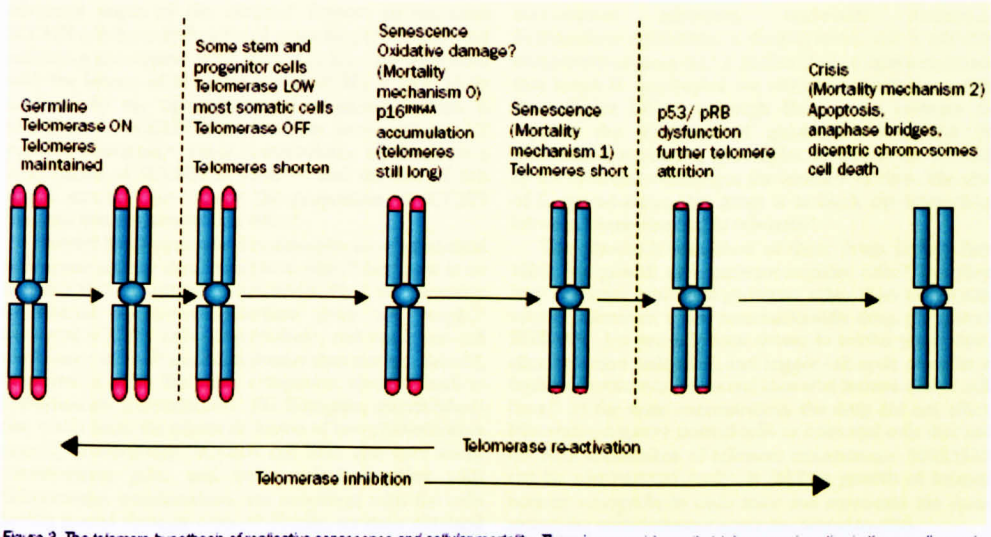

Figure 3. The telomere hypothesis of replicative senescence and cellular mortality. There is now evidence that telomerase is active in the germline and that it maintains the telomeres through successive generations. At some point during development, telomerase is downregulated or extinguished in most somatic human cells; from that time telomeric attrition is proposed to begin. Most carcinomas first encounter the telomere-length-independent mortality mechanism 0, which is bypassed by the loss of p16INK4A early in development of SCCHN. Telomere shortening continues and culminates in replicative senescence or mortality mechanism 1. The elimination of the p53 and pRB pathways by DNA tumour viruses or by somatic mutations in the pathways themselves allows the cells to tolerate short telomeres and divide further. In the continued absence of telomerase, this division results in further telomeric attrition until the telomeres become dysfunctional and the cells die as a consequence of mitotic failure precipitated by dicentric chromosomes and anaphase bridges. The reexpression or upregulation of telomerase adds TTAGGG repeats to the dysfunctional telomeres, renders them functional again and allows the cells to bypass crisis, or mortality mechanism 2. Conversely the inhibition of telomerase drives telomerase-positive cells back into crisis.

anaphase bridges form and result in much cell death.[35,36] This checkpoint on the pathway to immortality is known as crisis or mortality mechanism 2 (M2; figure 3).[33] From the unstable cultures arise cells that are generally telomerase positive, maintain their telomeres, and are immortal.[35] However, less commonly, telomerase-negative cells survive crisis and become immortal because they use a recombination-based telomere elongation mechanism known as ALT (alternative lengthening of telomeres).[37] Although this mechanism has not to date been demonstrated in SCCHN, its possible relevance to the successful treatment by telomerase inhibitors is discussed below. The role of telomerase deficiency in human cellular mortality is shown by the findings that the ectopic expression of telomerase can bypass both senescence[25] and crisis[26] in these models.

Telomere function and the development of cancer

The possibility of reconstituting telomerase in cultured human cells has enabled investigators to create human tumour cells with a small number of defined genetic changes, which is not possible in the absence of telomerase.[38] Thus, these observations establish telomerase activation as an important event in the conversion of a normal human cell to a malignant cell. In the telomerase-null mouse, the development of squamous cancer elicited by chemical carcinogens[39] is strongly repressed, and the development of other cancers is reduced in mice additionally deficient in the adenomatous polyposis coli (*Apc*) and *INK4A-/-* tumour suppressor genes.[40,41] However, when short telomeres are combined with p53 deficiency, there is an increased tumour yield[42] and tumours with cytogenetic features (unbalanced translocations, Robertsonian translocations, regional gains and losses) reminiscent of the human tumours are observed.[43] The generation of genetic instability by dysfunctional telomeres and the acceleration of cancer development are speculated to occur during a short period in which many human cancer cells will die as part of crisis.[44] Once telomeres become functional again, through the upregulation of telomerase, the genetic stability of the tumours is predicted to be restored. The concept that in human tumours the genetic instability generated during crisis contributes to the upregulation of telomerase is certainly plausible. The combination of short dysfunctional telomeres and p53 mutation is likely to exist in the latter stages of development of many human cancers,[29,45] and the inhibition of telomerase may accelerate the development of subsets of tumours that can use ALT mechanisms to maintain telomeres.

SCCHN immortality and the role of telomerase

The immortal phenotype can arise early in SCCHN progression[46] but generally is more common in the

advanced stages of the disease.[47] Almost all immortal SCCHN cells have dysfunctional genes for $p16^{INK4A}$, p53, and retinoic-acid-receptor β;[48,49] these markers are associated with the bypass of M1. The bypass of M2 is most likely achieved by the upregulation of telomerase, which is ubiquitous in SCCHN lines; there are no reports of ALT pathway activation.[48] These characteristics are found in a large subset of SCCHN in vivo,[5,48,47] and the size of this subset matches very closely the proportion of SCCHN tumours that are immortal in vitro.[47]

Normal basal squamous keratinocytes have substantial telomerase activity demonstrable in vitro,[50] but there is no evidence that the enzyme is functional. First, the telomeres of normal squamous epithelium from old people,[51] immortal SCCHN cells (unpublished), and squamous-cell carcinomas in vivo[52] are much shorter than normal. Second, immortal SCCHN lines have cytogenetic changes, such as Robertsonian translocations (KG Edington, unpublished) that result from the telomeric fusion of two chromosomes (usually acrocentrics). SCCHN cell lines also have many chromosomal gains and losses, which together with Robertsonian translocations, are consistent with the cells having passed through crisis.[43,44] Finally, we have obtained direct evidence of the existence in vivo of post-M1, pre-M2 SCCHN cells (unpublished). SCCHN cells with mutant p53, deleted INK4A, short telomeres, and low telomerase activity can be detected in vitro. Such cells show features of crisis, including dicentric chromosomes and anaphase bridges. The ectopic expression of telomerase in these cells reduces numbers of dicentric chromosomes and anaphase bridges and improves growth. Furthermore, some SCCHN tumours have high anaphase-bridge indices in vivo. Taken together, our findings are consistent with the idea that SCCHN cells reactivate telomerase after a long period of telomere shortening.

The very short telomeres of most SCCHN compared with the normal tissues of the body should ensure that, after the inhibition of telomerase, the cancer cells will die before the normal cells are affected.

Telomerase-based cancer therapies in SCCHN

There are several ways in which telomeres and telomerase could theoretically be exploited in the treatment of human cancer. First, when telomerase inhibition is achieved experimentally by various procedures, the phenotypic lag before death is always proportional to the starting telomere length.[16,53–56] Inhibition of telomerase by antisense[16] or dominant-negative[55,56] telomerase components, oligonucleotides,[54] or unknown suppressor genes,[56] drives many tumours back into M2 crisis and causes them to undergo apoptosis. The same manipulations do not affect cells that maintain their telomeres by means of the ALT pathway.[53] Furthermore, the killing of tumour cells through telomerase inhibition does not require the genes for p53 or INK4A to be intact,[53,56] which is important, given the frequency of the inactivation of these genes in human tumours. These studies show the usefulness of telomerase as an anticancer target.

There are now reports of the first drugs that can achieve telomerase inhibition.[4,57] Such drugs include reverse-transcriptase inhibitors, nucleoside analogues, isothiazolone derivatives, a rhodocyanine, and a catechin component of green tea.[4] A further class of agents are those that target G quadruplex (or tetraplex) structures rather than duplex DNA.[4] Although there is no evidence to support the existence of quadruplex structures in mammalian telomeres, quadruplex structures are formed by the G-strand overhang at the telomere in vitro. The aim of G-quartet-interacting drugs is to block the interaction between telomerase and its substrate.[4]

The drawback with most of these drugs is that they affect the growth of telomerase-negative cells,[58,59] so they may influence biological processes other than telomerase activity. However, a new non-nucleosidic drug, designated BIBR1532, has recently been shown to inhibit telomerase, elicit telomere reduction, and trigger cell-cycle arrest in a highly selective way, in several immortal human cancer cell lines.[57] At the same concentration, the drug did not affect telomerase-negative normal cells or immortal cells that use the ALT mechanism of telomere maintenance. BIBR1532 can be administered orally. It inhibits growth of human tumour xenografts in nude mice and represents the most promising antitelomerase agent discovered to date.

Although cryptic damage, in the form of telomere reduction, may be sustained by normal telomerase-positive cells, the available evidence suggests that on removal of telomerase inhibition, the telomeres return to their original length.[54] This evidence suggests that normal cells may be able to reset their telomere lengths once antitelomerase therapy has been withdrawn. This idea is also supported by the ability of senescent bovine cells to produce cloned animals with normal telomere lengths.[11]

A second approach that has been used is to exploit the absence of TERT transcription in most normal human cells and to express proapoptotic caspases under the control of the active TERT promoter in cancer cells. With this approach, a substantial cancer-specific cell kill has been reported.[60] However, the presence of almost equivalent amounts of TERT transcript in the basal layers of normal squamous epithelium and immortal keratinocyte lines[61] argues against the use of this approach in SCCHN.

A further approach has been to incorporate oligonucleotides encoding dysfunctional telomerase templates into cells.[62] In this strategy, only cells with functional telomerase incorporate the mutant sequences into telomeres to render them dysfunctional and only the telomerase-expressing cells die. This idea may be usefully applied to SCCHN (if the telomerase in the basal layer of squamous epithelium is dysfunctional) and has the added advantage of producing telomerase-dependent cell death in cancer cells with long average telomere repeat fragments.

As with many gene-therapy approaches, these strategies depend on the development of suitable gene-delivery systems.

Telomere dysfunction and radiosensitisation

A role of telomeres in chemosensitivity and radiosensitivity has lately emerged.[63–66] When telomerase is inactivated in mice by the targeted disruption of the *TERC* gene, the

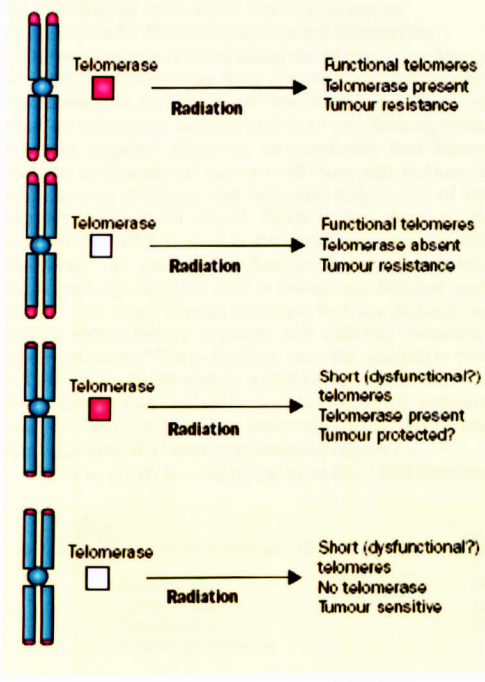

Figure 4. Dysfunctional telomeres sensitise cells to ionising radiation. The figure summarises how telomere length regulates the radiosensitivity of mouse cells engineered to lack telomerase and to have either short or long telomeres. The presence (red) or absence (white) of telomerase is indicated by the boxes, and the size of the telomeres by the red portions of the chromosomes. The results so far indicate that cells remain resistant to radiation even in the absence of telomerase, provided that the telomeres are long. The combination of an absence of telomerase and short telomeres results in telomere dysfunction and increased radiosensitivity. However, whether the presence of telomerase alone is enough to protect cells from radiation, even when the telomeres are short, is not clear.

telomeres progressively shorten with each generation until a radiosensitivity syndrome is induced, leading to accelerated mortality on exposure to ionising radiation.[46] These experiments showed that the radiosensitivity was related to telomere dysfunction rather than the mere absence of telomerase (figure 4). At the cellular level, crypt stem cells in the gastrointestinal tract and primary thymocytes in TERC-/- mice were shown to have increased rates of apoptosis. Furthermore, mouse embryonic fibroblasts had diminished clonogenic survival. This cellular-level radiosensitivity correlates with the persistence of complex chromosomal aberrations and retarded DNA repair kinetics, showing an intimate relation between intact, functioning telomeres and the cellular and whole-organism response to ionising radiation.[46]

Goytisolo and colleagues have also shown a correlation between telomere length and radiosensitivity.[45] They found that fifth-generation telomerase-deficient mice, which had telomeres 40% shorter than wild-type control animals, were hypersensitive to ionising radiation. The mice died of acute radiation poisoning in the gastrointestinal tract, lymphoid organs, and kidneys, and the telomerase-deficient animals showed greater chromosomal damage and higher numbers of apoptotic cells than the similarly irradiated control mice.

All these findings suggest that telomerase-inhibitor chemotherapy, producing shortened telomeres in cancer cells, should increase the radiosensitivity of the tumour selectively and therefore improve tumour kill. This proposition is very attractive, not least because this anticancer therapeutic strategy makes use of the already existing treatment infrastructure. Furthermore, it may allow use of lower doses of ionising radiation, thus achieving greater selective tumour kill with less severe side-effects of radiotherapy. However, the finding that telomere dysfunction can elicit rapid apoptosis via a p53-dependent pathway in mice[46] could present an obstacle to the radiosensitisation of SCCHN by telomerase inhibition, since virtually all SCCHN lines with high telomerase activity have lost p53 function.[48] Encouragingly, the situation is different in human cancer cell lines, where in many cases p53 is unnecessary for apoptosis when telomerase is inhibited.[32] Furthermore, the p53 status of SCCHN cultures does not influence their radiosensitivity.[47] However, the role of p53 in telomere-dysfunction-related phenotypes clearly needs to be examined in SCCHN.

Telomere dysfunction, chemotherapy, and the role of p53

Recent evidence from work in oncogene-transformed telomerase-deficient mouse cells has shown that telomere dysfunction rather than the lack of the telomerase enzyme itself is the main determinant of sensitivity of tumours to certain chemotherapeutic agents.[45] Telomerase-deficient transformed cells with short, dysfunctional telomeres were sensitised to killing by drugs inducing double strand breaks (doxorubicin, daunorubicin, and dactinomycin), but not drugs working through other mechanisms (cisplatin, etoposide, or fluorouracil). Sensitisation was associated with increased chromosomal fragmentation and fusion. Moreover, all the effects were reversed by the reintroduction of telomerase. This evidence of specific sensitisation of tumour cells highlights one of the most attractive features of the telomerase and telomere target. However, the effects were somewhat muted in mouse cells without wild-type p53. The introduction of a hammerhead ribozyme into human breast tumour cells and an immortal breast cell line produced cleavage of TERT mRNA and attenuation of telomerase activity,[44] resulting in sensitivity to doxorubicin. Furthermore, the introduction of telomerase into human fibroblasts reduced sensitivity to the same drug.[44] As in the first study, sensitivities to other cytotoxic drugs, such as cisplatin, were not affected.

These studies suggest that in certain situations the combined use of chemotherapy and telomerase inhibition may be effective against SCCHN, but the role of p53 in telomere-dysfunction-mediated chemosensitisation needs to be investigated.

ALT pathway and other mechanisms of resistance to telomerase-based therapies

Other mechanisms of maintaining the telomeres are known to exist, including one that depends on homologous recombination, in both yeast[48] and mammals.[49] Such cell lines are telomerase negative and have very heterogeneous telomere lengths.[49] However, we emphasise that human tumours and immortal tumour cell lines with features of ALT are not common and represent only 6–7% of the samples analysed to date.[49] Furthermore, there is no evidence that ALT is used to maintain untreated SCCHN telomeres.[48] By contrast, dysfunctional DNA mismatch-repair pathways facilitate ALT in telomerase-deficient yeast strains, and many human tumours that are resistant to various chemotherapy regimens lack efficient mismatch-repair pathways.[50] These findings raise the possibility that SCCHN tumours previously debulked by radiotherapy or chemotherapy may lack efficient mismatch-repair pathways and be predisposed to activating ALT after the administration of a telomerase inhibitor (figure 5).

There is, as yet, no evidence at all to show that tumours debulked by radiotherapy or chemotherapy can activate ALT more easily; unfortunately, mismatch-repair pathways have not been extensively investigated in SCCHN. However, the one published study did not find any significant selection for cells deficient in mismatch-repair pathways after chemotherapy.[51] The possibility that deficiency in these pathways may assist telomere maintenance by ALT is a key issue in combined use of antitelomerase and conventional therapies. Combination therapy is likely to be necessary to create telomere dysfunction in the cases of SCCHN with longer starting telomere lengths. The status of mismatch-repair pathways in SCCHN that have recurred after conventional therapy therefore needs to be examined before combination of telomerase inhibitors with such therapies can be considered.

Another possible means by which a tumour might be resistant to a telomerase inhibitor is by inactivating apoptosis mechanisms that act downstream of a critically short telomere.[1] There is some evidence that this process occurs after the experimental inactivation of telomerase in

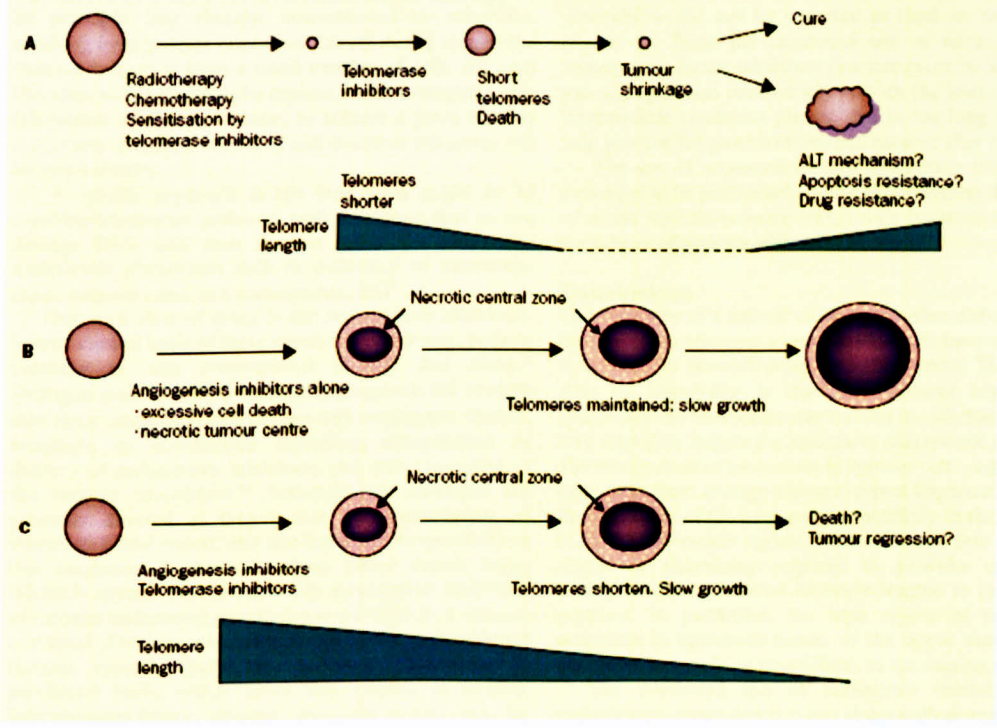

Figure 5. Telomerase inhibitors in combination therapy. After shrinkage by radiotherapy (a), the tumour would grow for a variable period in the presence of a telomerase inhibitor until the gradual attrition of the telomeres provoked apoptosis and resulted in substantial cell death. Whether ALT or other resistance mechanisms might contribute to relapse at this point is still debatable. (b) The limitations to the use of angiogenesis inhibitors alone. The inhibitors slow the growth of the tumour by denying the more central cells a blood supply. However, the tumour expands owing to the viable outer rim. (c) In this situation, the slow-growing tumour undergoes telomeric attrition during the multiple divisions required to replace the cells dying in the necrotic centre. The hypothesis is that the tumour cells will die as a consequence of telomere dysfunction. This strategy has the advantage of using two complementary and very specific novel approaches to cancer treatment.

certain human cells, immortalised by adenovirus,[55] but not so far in human cancer cells.[53,56] Nevertheless, we need to understand in more detail the mechanisms governing apoptosis after telomere dysfunction and how this process leads to cell death during crisis.

Can telomerase inhibition increase the likelihood of second primary tumours?

Second primary tumours remain a substantial cause of treatment failure in SCCHN.[1] As detailed above, there is now evidence that a combination of telomere and p53 dysfunction could contribute to the progression of cancer.[34,40-44] Many p53-deficient clones exist at distant sites in the normal oral mucosa of patients with SCCHN, and these clones may contribute to cases of second primary tumours.[72] Whether the inhibition of telomerase over a long period increases the progression of second primary tumours in SCCHN patients remains to be seen.

Telomerase inhibitors in combination therapy

In principle, any therapy, conventional or otherwise, resulting in large-scale tumour-cell death would require the tumour to regrow from a small number of cells. All such therapies would therefore be expected to be synergistic with telomerase inhibitors, because, to achieve a given tumour mass more divisions will occur and therefore telomeres will be much shorter

A specific approach in the long term might be to combine telomerase inhibitors with new drugs that do not damage DNA and thus do not select for potentially undesirable phenotypes such as deficiency of mismatch-repair pathways and, as a consequence, ALT.

One such class of drugs is the angiogenesis inhibitors. Several clinical trials of these agents are under way, both in combination with conventional therapy and alone.[73] Strategies used to inhibit tumour angiogenesis fall broadly into three categories: interference with angiogenic ligands, receptors, or downstream signalling; upregulation or delivery of endogenous inhibitors; and direct targeting of the tumour vasculature.[74] Antiangiogenic strategies are generally directed at targets that are characteristic of immature blood vessels, and this feature gives specificity to this anticancer treatment, mature native vessels being relatively spared. One recent study investigated inhibition of vascular endothelial growth factor 2 (VEGF2); it showed a reversal of resistance to radiotherapy in two radioresistant tumour types.[75] There are, however, limitations in preclinical trials, which mean that caution is needed. Subcutaneous human tumour xenografts in mice may be less robust than human tumours in vivo, and the endpoint commonly used in preclinical studies is tumour regression rather than elimination. With tumour progression, a wider array of angiogenic molecules is produced. Hence, any therapy directed against a single agent (eg, VEGF) may produce selection of cells expressing another molecule (eg, interleukin 8). Agents against many targets used synchronously may be the most effective strategy.

The available evidence suggests that angiogenesis inhibitors can limit the expansion of tumours but do not kill the tumour completely (figure 5). The combined use of angiogenesis inhibitors and radiation is more effective than either alone in human xenografts,[76] so if sufficient targeting can be applied to tumours, greater success might be expected with angiogenesis-factor inhibitors in the future. Telomerase inhibitors are complementary to angiogenesis inhibitors in that they kill the tumour very efficiently but generally would not be expected to limit its size at first (figure 5). Thus the combined use of telomerase and angiogenesis-factor inhibitors (see comment by Shay[4]) may provide optimum tumour kill in both the immediate and intermediate treatment phases, and in the long term may help prevent the growth of residual tumour after therapy.

The use of angiogenesis and telomerase inhibitors is envisaged to be particularly useful in preventing the growth of occult micrometastases, which are a common reason for the relapse of patients with solid tumours.

Search strategy and selection criteria

Published and unpublished data for this review were collated by searches of Medline and Web of Science and from review of work presented at a recent international cancer meetings (American Association of Cancer Research, 2001) up to and including January, 2002. The search terms "telomerase", "telomeres", "cancer", and "head and neck cancer" were used. Only papers published in English were included. We have used recent reviews where possible, so that we could comply with the journal's policy on total number of references. Where more than one reference is relevant, we have used the first published.

Conclusions

The discovery of a mitotic clock and further elaboration of the role of the telomere in mammalian cells have stimulated many ideas in research connected with cancer. The current state of knowledge is that the telomere length, and specifically the telomerase enzyme and its cofactors, provide very attractive targets for anticancer therapeutic strategies. The reactivation of telomerase in tumour cells and, in many cases, very short average telomere repeat fragments, provide the possibility of the long-sought specificity in the targeting of chemotherapeutic agents. Further insight into the exact degree of shortening required to provoke crisis and correlation with observed telomere lengths in cancers are required. In particular, the high degree of telomerase activation in squamous cancer of the upper aerodigestive tract promises exciting possibilities in the coming years.

The combined use of telomerase inhibitors with radiotherapy, more selective new drugs such as angiogenesis inhibitors, or both, to kill cancer cells selectively is an exciting prospect. The publication of a report on the first highly selective antitelomerase drug[77] brings us closer to the point where the ideas discussed in this review can be realised and tested in vitro and then in vivo. Such new initiatives, if successful, may finally herald a new era in which the prognosis for SCCHN may finally improve.

Acknowledgments

We thank Paul Harrison and John Wyke for their detailed reading of the review and constructive comments. JAMcC is the recipient of a

Scottish Council for Postgraduate Medical and Dental Education/ Chief Scientist Office Clinical Research fellowship. KEG and EKP are supported by the Cancer Research UK.

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