G_2 chromosomal radiosensitivity in childhood and adolescent cancer survivors and their offspring

A thesis submitted to the University of St Andrews

for the degree of Ph.D. in the Faculty of Science

July 2007

Gillian B Curwen

Department of Genetics, Westlakes Research Institute.

Faculty of Science, University of St Andrews.

TABLE OF CONTENTS

DECLARATIONS

I, Gillian Curwen, hereby certify that this thesis, which is approximately 41,500 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date …….. Signature of candidate ……..…

I was admitted as a research student in September 2001 and as a candidate for the degree of Ph.D. in September 2002; the higher study for which this is a record was carried out in the University of St Andrews between 2001 and 2007.

Date …… Signature of candidate …….

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date ……. Signature of supervisor ……

Date ……. Signature of supervisor ……....

COPYRIGHT

In submitting this thesis to the University of St Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker, that my thesis will be electronically accessible for personal or research use and that the library has the right to migrate my thesis into new electronic forms as required to ensure continued access to the thesis. I have obtained any third-party copyright permissions that may be required in order to allow such access and migration.

Date ……..…… Signature of candidate ……

ACKNOWLEDGMENTS

The work reported in this thesis was funded by and carried out at Westlakes Research Institute. I would like to thank all staff at the Institute for their help and encouragement over the years and give a special thanks to Drs Valerie Smart, Craig Wilding and Gwen Rees, Professor Jan Tawn and Mrs Caroline Whitehouse for their invaluable advice. I would also like to thank Dr Peter Bryant, St Andrews University, for his supervision and patience. In addition, I would like to thank all international collaborators, Miss Annette Savage for advice on drawing figures, Mr Keith Binks for statistical advice, Dr Valerie Smart for help with chromatid aberration scoring and the Danish families who donated blood samples, without whom this study would not have been possible.

ABSTRACT

It is increasingly recognised that individual risk of cancer may be related to genetically determined differences in the ability of cells to identify and repair DNA damage. Cell cycle based assays of chromosomal radiosensitivity provide the greatest power for discriminating differences in response to DNA damage and it has been suggested that individuals who are genetically susceptible to cancer show increased chromosomal radiosensitivity. The relationship between chromosomal radiosensitivity and early onset cancer was investigated in a population of Danish survivors of childhood and adolescent cancer and a control group comprising of their partners using the G_2 assay of chromosomal radiosensitivity. Heritability was also examined in the offspring.

No significant differences in radiosensitivity profiles were found between partner controls and either the cancer survivors or offspring. However, when compared to the Westlakes Research Institute control population, significant differences were observed with the cancer survivors ($P = 0.002$) and offspring ($P < 0.001$), supporting an association of chromosomal radiosensitivity with cancer predisposition. Heritability studies suggested the majority of phenotypic variance of chromosomal radiosensitivity was attributable to a putative major gene locus with dominant effect.

Since G_2 chromosomal radiosensitivity indirectly measures the ability of cells to repair DNA damage induced by ionising radiation exposure, variants in DNA repair genes may explain inter-individual variation observed. Sixteen polymorphisms in nine genes from four DNA repair pathways were investigated. Genotype frequencies at the Asp148Glu polymorphism were associated with childhood cancer in survivors. Analysis of variance

and FBAT analysis suggested significant associations at both the Thr241Met and Ser326Cys polymorphism sites with G_2 radiosensitivity, but neither remained significant after multiple-test adjustment.

This study invites further exploration of the predictive capacity of G_2 chromosomal radiosensitivity in cancer predisposition. Clearly, further work is needed to correlate radiosensitivity with genetic polymorphisms, which may underlie cancer susceptibility and variation in radiosensitivity.

CHAPTER 1

Introduction

Aims of study

Radiosensitivity is the term used to describe the degree of reaction to ionising radiation displayed by tissues or cells and classically has been assessed by monitoring end-points such as cell-survival and chromosomal damage. Initial links between radiosensitivity and cancer susceptibility arose from studies of patients with chromosome instability syndromes such as ataxia telangiectasia (AT) (Bender *et al.*, 1985; Parshad *et al.*, 1985; Sanford *et al.*, 1990; Shiloh *et al.*, 1989). Today, much of the research in this area is focused on the implementation of chromosomal radiosensitivity as a biomarker for susceptibility to many other forms of cancer (Baria *et al.*, 1999; Riches *et al.*, 2001; Scott *et al.*, 1994a; Terzoudi *et al.*, 2000).

The study described in this thesis aimed to further investigate the putative relationship between cancer susceptibility and chromosomal radiosensitivity by examining peripheral blood samples from childhood and adolescent cancer survivors, their partners and their offspring using the G_2 assay of chromatid breakage (Scott *et al.*, 1994a). Individual variation in chromosomal radiosensitivity was examined to test the hypothesis that a greater number of childhood cancer and adolescent survivors would exhibit enhanced radiosensitivity in comparison with their partners who served as the control group. In addition, the response of their offspring was investigated for evidence of an inherited radiosensitive phenotype.

Variation in individual cellular or chromosomal radiosensitivity is thought to result from differences between individuals, in the ability of their cells to recognise and repair damaged DNA, with this being mediated through specific mutations or polymorphisms in genes involved in maintaining the integrity of the genome. This genetic variation may also be responsible, in part, for differences in cancer susceptibility between individuals (Mohrenweiser & Jones, 1998). Several complex pathways exist to identify and repair lesions in DNA that have been introduced either during replication or by exogenous or endogenous DNA damaging agents. Examples of these systems are those responsible for rejoining DNA double-strand breaks; the homologous recombinational repair system (Thompson & Schild, 2001) and the more important (in the case of mammalian cells) non-homologous end-joining system (Jackson, 2002). Mutations in the genes involved in these systems compromise the ability of a cell to respond to DNA damage and could lead to chromosomal radiosensitivity and possibly cancer through the accumulation of unrepaired or misrepaired DNA lesions (Sarasin, 2003). In this study, besides the assessment of chromosomal radiosensitivity by the G_2 assay, a range of polymorphisms associated with a panel of candidate DNA damage recognition and repair genes were also investigated in the above-mentioned Danish 'trio' groups of childhood and adolescent cancer survivors, their partners and offspring.

Cancer

Cancer incidence and causes

Cancer is a leading cause of death in the western world. One in three people will be diagnosed with some form of cancer during their lifetime. In the UK, more than 275,000 new cases of cancer were registered in 2002 and despite advances in diagnosis and

treatment, over 153,000 people died from cancer in 2004. There are over 200 different types of cancer but the four major types, breast, lung, large bowel (colorectal) and prostate, account for over half of all cases diagnosed. Breast cancer is the most common cancer in the UK. Over one fifth of all cancer deaths are from lung cancer, followed by bowel and then cancer of the breast. Cancer is a disease that affects mainly older people, with 64% of cases diagnosed in those aged 65 and over, and more than 75% of deaths from cancer occurring in the same age group. As the average life expectancy in the UK has almost doubled since the mid-nineteenth century, the population at risk of cancer has also grown concomitantly and over the last 25 years the incidence of all cancers has increased by 24%. Other types of cancers are more common in the young and are hardly ever seen in adults. Approximately 1,500 new cases of childhood cancer are diagnosed each year in the UK, with leukaemia being the most common representing one third of all cases (refer to **http://info.cancerresearchuk.org/cancerstats/**).

Cancer is a complex and large family of diseases, and carcinogenesis (the conversion of a normal cell into a cancer cell) is a multistep process (Sarasin, 2003). Ultimately, cancer is a disease of abnormal gene expression. This altered gene expression is thought to occur through a number of mechanisms, including damage to DNA and abnormal gene transcription or translation (Sarasin, 2003). In many cases, the causes of cancer are not clearly defined. However, both external (environmental chemicals and radiation) (Montesano & Hall, 2001) and internal factors (immune system defects or genetic predisposition) (Peto & Houlston, 2001) usually play a role, and several such factors may act together to initiate and promote carcinogenesis.

Susceptibility to cancer

Approximately 80% of most common cancers are sporadic, an additional 10-15% are referred to as familial with susceptibility caused by mutations in one or more low penetrance genes, gene-environment interactions, or both, and 5–10% are inherited and arise due to highly penetrant mutations (e.g. *BRCA1* and *BRCA2*) (Nagy *et al.*, 2004). Some of the mechanisms by which inherited mutations in certain genes cause a high cancer risk are already known and help provide an insight into the evolution of cancer in non-susceptible individuals (Peto & Houlston, 2001). To date, the majority of the cancer predisposing genes discovered have been found to be highly penetrant (refer to Table 1.1) but fortunately are too rare to cause more than a few percent of most cancer types, for example, the *ATM* gene which causes AT and the *BRCA1* and *BRCA2* genes which cause hereditary breast ovarian cancer syndrome (HBOC) (Nagy *et al.*, 2004). Penetrance of the genes is also variable. For example, *BRCA1* and *BRCA2* are highly predisposing in heterozygotes (homozygotes are embryonic lethal) whereas *ATM* is only mildly penetrant (with regard to cancer susceptibility) in obligate heterozygotes; only the homozygotes show the full gene penetrance (Swift *et al.*, 1986). Evidence is now emerging that a high proportion of cancers arise due to genetic susceptibility but the relevant genes or gene combinations are of low penetrance and therefore do not cause large multiple-case cancer families. If such genes do indeed cause a substantial number of cancers, their identification would be of great importance and the ability to recognise those individuals with an elevated susceptibility would be beneficial through earlier identification and treatment of the cancer resulting in enhanced prognosis.

Table 1.1: Some highly penetrant cancer syndromes.

Syndrome	Gene(s)	Population incidence	Penetrance
Ataxia telangiectasia	*ATM*	1/30,000 to 1/100,000	100%
Bloom syndrome	*BLM*	Unknown, rare	100%
Carney complex	*PRKRA1A*	Rare	Unknown
Cowden syndrome	*PTEN*	1/200,000	90–95%
Familial adenomatous polyposis	*APC*	1/5,000 to 1/10,000	100%
Fanconi anaemia	*FANCA, B, C, D_1, D_2, E, F, G, I, J, L, M, N*	1/360,000	100%
Hereditary breast-ovarian cancer syndrome	*BRCA1, BRCA2*	1/500 to 1/1000	Up to 85%
Hereditary nonpolyposis colon cancer	*MLH1, MSH2,6,PMS1, 2*	1/400	90%
Li-Fraumeni syndrome	*TP53*	Rare	90-95%
Multiple endocrine neoplasia type 1	*MEN1*	1/100,000	95%
Multiple endocrine neoplasia type 2	*RET*	1/30,000	70–100%
Nijmegan breakage syndrome	*NBS1*	Rare	100%
Peutz-Jeghers syndrome	*LKB1*	1/200,000	95–100%
Retinoblastoma	*RB*	1/13,500 to 1/25,000	90%
Xeroderma pigmentosum	*XPA,B, C, D, E, F, G, V, ERCC2, 3, 4, 5,*	1/1,000,000	100%

Detecting cancer susceptibility

As mentioned above, there are many different pathways and mechanisms responsible for cancer susceptibility in the general population and due to their low penetrance, identifying them is not straightforward. Unless the specific gene involved in causing the defect is characterised, which is not always possible for low penetrance genes, gene combinations and gene-environment interactions, other methods must be relied upon (Peto & Houlston, 2001). Indeed, the only successful mechanism to date for identifying low penetrance genes has been the analysis of polymorphisms at candidate loci (refer to Table 1.2) although again, the effect of such polymorphisms in combination with each other and with environmental risk factors will only ever be known when substantial data is available for very large numbers of patients and controls. With respect to this it is clear that alternative methods must be sought and our ability to do this is increasing rapidly due to powerful analytical tools and the almost complete genome sequence information from human and model organisms.

Such alternative methods include DNA microarray technology which has had a huge impact in the field of cancer research by allowing mass gene expression studies to be undertaken using high-throughput screening. Microarrays are created by robotic machines that arrange minuscule amounts of thousands of gene sequences on a single microscope slide. Researchers have a database of over 40,000 gene sequences that they can use for this purpose enabling the transcript level of thousands of genes to be measured simultaneously. If a particular gene is very active, it produces many molecules of messenger RNA, which hybridise to the DNA on the microarray and generate a very bright fluorescent area. Genes that are somewhat active produce fewer mRNAs, which results in dimmer fluorescent spots. If there is no fluorescence, none of the messenger molecules

have hybridised to the DNA, indicating that the gene is inactive. Researchers use this technique to examine the activity of various genes at different times and so permit the identification of differentially regulated genes. In this way, the transcriptional characteristics of tumours can be assessed and mutations in potential low penetrance cancer genes discovered. In addition, microarray technology can also be used as a high throughput method for genotyping SNPs, enabling variation at multiple loci to be analysed in a single experiment and the presence of all genotypic combinations assessed. In this way, the identification of a particular genomic region associated with a particular trait or pathway can be targeted.

Other analytical tools include chromosome breakage assays such as the micronucleus and comet assays, or fluorescence *in situ* hybridisation, which allows the rapid detection and genomic localisation of structural aberrations in metaphase spreads. Restriction landmark genome scanning, comparative genomic hybridisation (CGH), high-throughput quantitative PCR and molecular 'subtraction' techniques such as representational display analysis allow the rapid detection and genomic localisation of aberrations and mutations throughout tumour genomes.

Table 1.2: Genetic polymorphisms for low penetrance cancer susceptibility loci.

Adapted from Peto & Houlston (2001).

Class-Locus	Cancer	Putative mechanism
Metabolic polymorphisms		
CYP1A1	Lung, breast, colorectal, Uterine	Altered metabolism
CYP1A2	Bladder, colorectal	Altered metabolism
CYP2D6	Lung, liver	Altered metabolism
GSTM1	Lung, bladder, breast, Gastric, colon, Head and neck, uterine	Altered metabolism
GSTT1	Colorectal, larynx, brain	Altered metabolism
NAT2	Bladder, colon, liver	Altered metabolism
Androgen receptor	Prostate	Altered metabolism
MTHFR	Colorectal, uterine	Methylation status
Tumour suppressor genes		
APC-I1307K	Colorectal	Hypermutability
DNA damage response genes		
ATM	Breast	Genomic instability
Proto-oncogene polymorphisms		
H-ras –VNTR	Colorectal, breast, lung, Bladder, leukaemia	Altered transcription

Cancer susceptibility and chromosome breakage syndromes

The classical chromosome breakage syndromes such as ataxia telangiectasia (AT) and Fanconi anaemia (FA) are autosomal recessive disorders that share elevated levels of spontaneous chromosome breakage and varying degrees of heightened cancer predisposition. Chromosome breakage is also associated with several other highly penetrant hereditary cancer predisposing syndromes leading to the suggestion that genetic instability may be a common mechanism by which the probability of oncogenesis is increased (Hsu, 1983). Accepting this, a correlation between cancer susceptibility and chromosome breakage could even be expected in the normal population. The unusually high frequencies of spontaneous and induced chromosome breakage observed in these syndromes are associated with a variety of DNA repair or processing defects, which are in turn, important molecular defence systems against agents that cause cancer. With respect to this, assays measuring breakage have had wide spread use as potential biomarkers for cancer susceptibility.

Xeroderma pigmentosum (XP) is another example of an autosomal recessive chromosome breakage syndrome characterised by chromosome instability and cancer predisposition. XP differs from the classical syndromes in that cells from sufferers have normal spontaneous breakage rates (Arlett, 1986). It is not until after exposure to UV light that chromosome aberration frequencies in XP cells increase at an abnormal rate and for this reason, XP is referred to as a 'conditional chromosome breakage syndrome'. The primary defect of this disorder involves components of the DNA excision repair pathway (Cleaver, 1968), specifically a deficiency in the excision of ultraviolet induced thymine dimers, the diagnosis of which can be made by measure of DNA synthesis using tritiated thymidine in place of normal thymidine in growth media of test cells. The amount of radioactive

thymidine incorporated into the DNA is a measure of the amount of nucleotide excision repair undertaken.

The example of XP provides clear evidence of the relationship between DNA repair and chromosome instability and, since it is estimated that at least 120 genes are involved in DNA repair (Hickson & Harris, 1988), a more extensive investigation of the association between chromosomal sensitivity after exposure to an appropriate mutagen and cancer susceptibility is warranted. This may help lead to other mechanisms for the phenotypic identification of low penetrance genes of this type.

G_2 chromosomal radiosensitivity and cancer susceptibility

Extensive research, often with controversial outcomes has been undertaken on the chromosomal response to X-irradiation and its relationship to cancer susceptibility. For example, numerous studies carried out by Katherine Sanfords group at the National Cancer Institute (NCI) in Bethesda, Maryland, have reported elevated chromosomal radiosensitivity in many of the cancer prone syndromes (refer to Table 1.3). These investigations involved X-irradiating exponentially growing cells and monitoring chromatid damage in cells entering metaphase 0.5 to 1.5 hours later, in order to obtain a measure of chromosomal radiosensitivity of cells which were in the G_2 phase of the cell cycle at the time of irradiation.

Of particular significance was the observation of enhanced G_2 chromosomal radiosensitivity in heterozygote carriers of the recessively inherited cancer prone syndrome AT and in approximately 5% of normal individuals (Sanford *et al.*, 1989). These findings generated much interest in the G_2 assay of chromosomal radiosensitivity in cancer patients

and cancer prone conditions but attempts to confirm the findings were met with mixed success (refer to Table 1.4 and Table 1.5 for references). After several attempts at reproducing this data, it was concluded that only AT and NBS unambiguously display elevated chromosome radiosensitivity, while the other cancer prone genetic disorders show less dramatic increases following *in vitro* radiation exposure (ICRP, 1998).

Table 1.3: Cancer prone syndromes displaying elevated G_2 chromosomal radiosensitivity as attributed by the NCI group.

Disorder	Reference
Ataxia telangiectasia homozygotes	(Parshad *et al.*, 1983; Sanford *et al.*, 1990; Sanford *et al.*, 1995).
Ataxia telangiectasia heterozygotes	(Parshad *et al.*, 1985; Sanford *et al.*, 1990).
Basal cell nevus syndrome	(Sanford *et al.*, 1987).
Bloom syndrome	(Parshad *et al.*, 1983).
Chronic ulcerative colitis	(Sanford *et al.*, 1997).
Down's syndrome	(Sanford *et al.*, 1995; Sanford *et al.*, 1993).
Familial breast cancer	(Parshad *et al.*, 1996).
Familial dysplastic naevus syndrome	(Sanford *et al.*, 1997; Sanford *et al.*, 1987).
Familial polyposis coli	(Parshad *et al.*, 1983).
Fanconi's anaemia	(Parshad *et al.*, 1983).
Familial retinoblastoma	(Sanford *et al.*, 1989; Sanford *et al.*, 1996).
Gardner's syndrome	(Parshad *et al.*, 1983; Takai *et al.*, 1990).
Hereditary cutaneous malignant melanoma	(Sanford *et al.*, 1987).
Li-Fraumeni syndrome	(Parshad *et al.*, 1993a).
Wilms' tumour	(Sanford *et al.*, 1989).
Xeroderma pigmentosum homozygotes	(Parshad *et al.*, 1983; Price *et al.*, 1991; Sanford *et al.*, 1989).
Xeroderma pigmentosum heterozygotes	(Parshad *et al.*, 1990; Sanford *et al.*, 1989).

Table 1.4: Results obtained using the G_2 chromosomal radiosensitivity assay in lymphocytes. Taken from Scott (1994).

Individuals tested	Sensitive individuals[1] / total individuals
Controls	7 / 74
Ataxia telangiectasia homozygotes	12 / 12
Ataxia telangiectasia heterozygotes	28 / 28
Basal cell nevus syndrome	6 / 7
Bloom syndrome homozygotes	3 / 5
Bloom syndrome heterozygotes	2 / 2
Familial breast cancer (*BRCA1* linked)	1 / 4
Familial polyposis coli	1 / 2
Fanconi's anaemia homozygotes	2 / 2
Fanconi's anaemia heterozygotes	3 / 3
Hereditary retinoblastoma	1 / 1
Li-Fraumeni syndrome	5 / 5
Nijmegan breakage syndrome homozygote	1 / 1
Nijmegan breakage syndrome heterozygote	0 / 1
Rothmund-Thompson syndrome	1 / 1
Sporadic breast cancer	21 / 50
Xeroderma pigmentosum homozygotes	11 / 12
Xeroderma pigmentosum heterozygotes	7 / 8

[1] Individuals with a G_2 chromosomal radiosensitivity within the AT heterozygote range were classified as sensitive.

Table 1.5: Studies investigating G_2 chromosomal radiosensitivity in cancer susceptibility conditions.

Condition	Normal sensitivity[1]	Elevated sensitivity[2]
Aplastic anaemia		(Darroudi *et al.*, 1995)
AT heterozygotes	(Bender *et al.*, 1985)	(Scott *et al.*, 1994a; Shiloh *et al.*, 1989; Shiloh *et al.*, 1986)
AT homozygotes		(Bender *et al.*, 1985; Natarajan & Meyers, 1979; Shiloh *et al.*, 1986)
Bloom's syndrome	(Tice *et al.*, 1978)	(Aurias *et al.*, 1985; Kuhn, 1980)
Fanconi's anaemia	(Darroudi *et al.*, 1995)	(Bigelow *et al.*, 1979)
Li-Fraumeni syndrome		(Mitchell & Scott, 1997)
Lymphoma		(Darroudi *et al.*, 1995)
Nijmegan breakage syndrome		(Jaspers *et al.*, 1988)
Other familial cancers	(Bender *et al.*, 1988)	
Retinoblastoma	(Zampetti-Bosseler & Scott, 1981)	
Rothmund-Thompson syndrome		(Kerr *et al.*, 1996)
XP homozygotes	(Darroudi *et al.*, 1995)	

[1, 2] Normal and elevated sensitivity designated on the basis of standards defined within individual studies.

Cell cycle control, DNA repair and variation in radiosensitivity

The modern concept of cell cycle was discovered at the beginning of the 1950s and revealed the S-phase (DNA synthesis phase) to be separated from the mitotic phase by two 'gap' phases termed G_1 and G_2 (Howard & Pelc, 1953). In the early 1960s, the ability to produce a population of synchronised cells led to the discovery of large variation in radiosensitivity throughout the cell cycle and linked the cell cycle to DNA repair (Terasima & Tolmach, 1961; Terasima & Tolmach, 1963a; Terasima & Tolmach, 1963b). It took another 30 years before a major breakthrough between cell cycle checkpoints in response to DNA damage was fully developed (Hartwell & Weinert, 1989).

The response of an unsynchronised cell population following exposure to ionising radiation can be affected by a number of physical and biological factors. However, it is the processes that govern the cell cycle, DNA damage recognition and repair, and apoptosis that have the biggest impact on cell survival. To preserve genetic integrity, precise mechanisms monitor the progress of cells through the cycle (Zhou & Elledge, 2000). Following DNA damage, a series of checkpoints arrest the cell cycle in either G_1, S or G_2 phases to allow repair. The S-phase checkpoint halts the replication of damaged DNA (Falck *et al.*, 2002), whilst the checkpoints at G_1/S and G_2/M allow time to repair damaged DNA prior to entry into S and M phases, respectively. The latter two checkpoint pathways also function to control the activation of DNA repair pathways and movement of DNA repair proteins to the sites of DNA damage (Zhou & Elledge, 2000). DNA damage can be divided into several general classes which include dimers, adducts, oxidative base damage, intra- and inter-strand crosslinks, DNA-protein crosslinks, DNA single strand breaks (SSBs) and DNA double strand breaks (DSBs). In order to protect the integrity of

the genome, a number of different specialised DNA repair processes are invoked by cells, each being specific to the lesion type. Figure 1.1 illustrates DNA damage, repair mechanisms and consequences. Damaged DNA bases are repaired chiefly by nucleotide excision repair (NER) which removes UV induced pyrimidine dimers and bulky DNA adducts associated with chemical exposures; base excision repair (BER) which directly processes damaged bases such as oxidised bases, the most common spontaneous damage and thirdly, mismatch repair (MMR) which repairs replication errors. The most damaging lesions to cell survival are DNA DSBs and these are often associated with exposure to ionising radiation. DNA DSBs are also the principal initiating lesions leading to chromosome and chromatid aberrations (although inadequate DNA SSB repair can also act as a precursor to DNA DSBs). Processes known as homologous recombination repair (HR) and non-homologous end-joining (NHEJ) (refer to Figure 1.2) are required for repair of DNA DSBs (Valerie & Povirk, 2003). HR depends on DNA homology and involves swapping equivalent regions of DNA between sister chromatids or homologous chromosomes and restores the original sequence that existed prior to the damage. NHEJ uses little or no sequence homology thus sacrificing the original DNA sequence. During NHEJ, the ends of a break are modified by the addition and deletion of nucleotides and then ligated to restore the broken chromosome. The contribution of each mechanism in DNA DSB repair is not fully understood and it is not clear whether the two pathways compete or co-operate to repair DNA DSBs (Allen *et al.*, 2003). It is likely that HR is more important during late S and G_2 phases of the cell cycle when sister chromatids are present, whereas NHEJ is more prominent during G_1 and early S-phases. Indeed, many of the proteins involved in DNA damage recognition, cell cycle checkpoint activation and DNA repair are common in each process suggesting an integrated defence mechanism against chromosome damage.

Variation in radiosensitivity throughout the cell cycle has been studied widely (Biade *et al.*, 1997; Schneiderman & Schneiderman, 1984; Schneiderman *et al.*, 1983) with researchers concluding four main points as stated by Sinclair (1968); mitotic cells are generally the most radiosensitive; there is usually a resistant period in G_1 which declines towards S-phase; resistance increases during S-phase and is maximum towards the latter part of the phase; G_2 phase is almost as sensitive as mitosis. The first three conclusions have been unequivocally confirmed although for the latter there is somewhat conflicting evidence but in all studies, researchers have concluded that cell cycle checkpoints correlate with variation in cell cycle radiosensitivity. However, researchers have also showed that defects in cell cycle checkpoint activity do not always affect radiosensitivity (Kastan & Lim, 2000; Slichenmyer *et al.*, 1993) and there is stronger evidence for defects in repair pathways leading to radiosensitivity than checkpoint deficiencies (Kuhne *et al.*, 2004; Shen *et al.*, 1998). Deficiencies in a variety of DNA repair pathways are therefore potential contributors to enhanced G_2 chromosomal radiosensitivity.

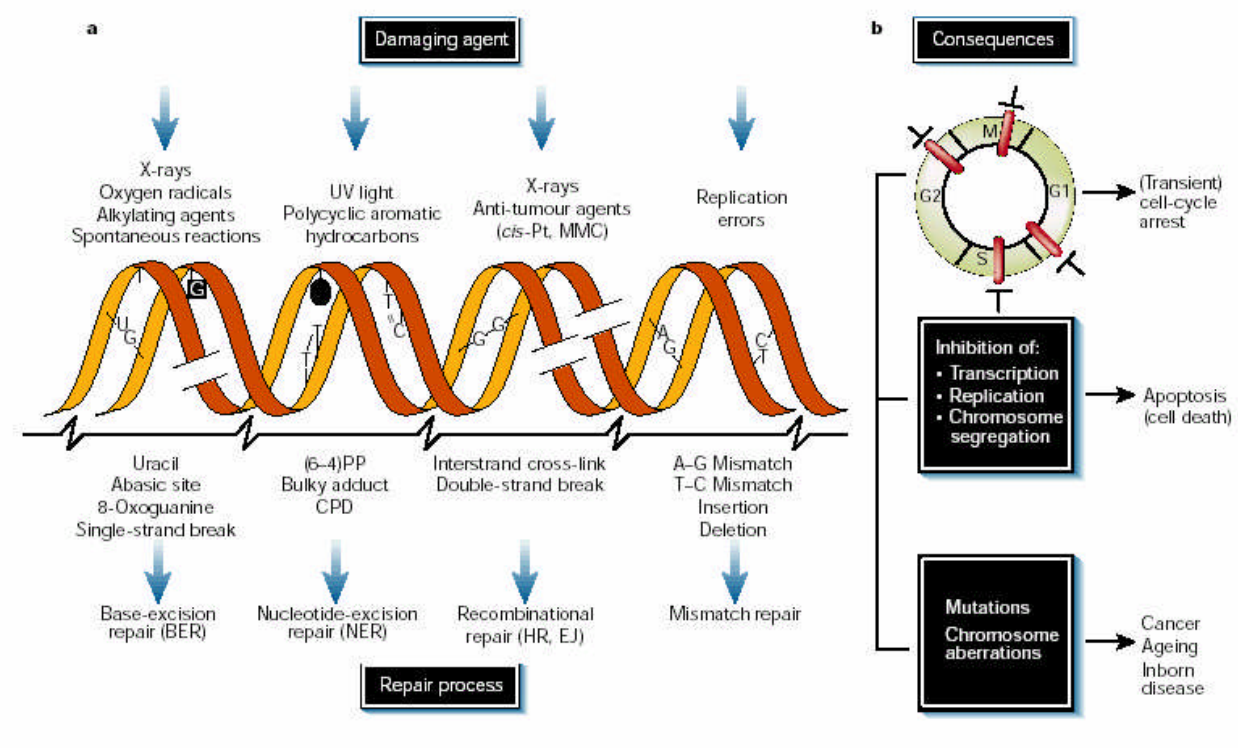


Figure 1.1: DNA damage, repair mechanisms and consequences.

a. Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); most relevant DNA repair mechanism responsible for the removal of the lesions (bottom).

b. *Acute effects of DNA damage on cell cycle progression, leading to transient arrest in the G_1, S, G_2 and M phases (top), and on DNA metabolism (middle). Long term consequences of DNA damage (point mutations affecting single genes or chromosome aberrations which may involve multiple genes) and their biological effects (bottom).*

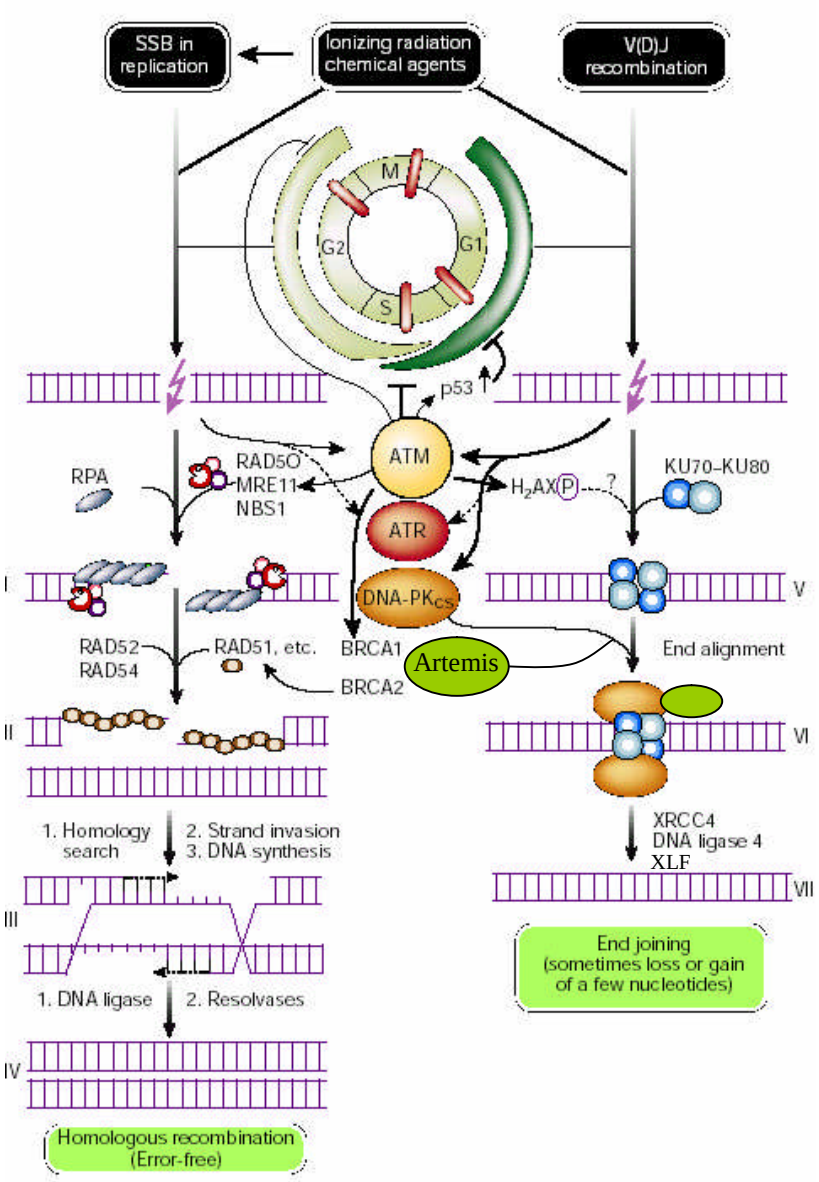
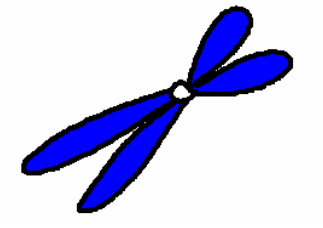


Figure 1.2: Mechanisms of homologous recombination (left) and non-homologous end joining (right). I. Strand invasion by RAD50/MRE11/NBS1 complex. II. RPA facilitated assembly of RAD51 nucleoprotein filament. III. RAD51 exchanges the single strand with the same sequence from a double stranded DNA molecule. Intact copy used to repair broken ends by DNA synthesis. IV. Holliday junctions resolved by resolvases. V-VII. Ends of a DSB linked together (without template) using KU70/80 complex and DNA-PK$_{CS}$-Artemis complex, followed by ligation by XRCC4-ligase4-XLF. Figure adapted from Weterings & Gent (2004).

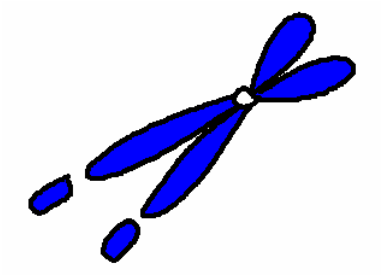
Mechanisms of G_2 chromosomal radiosensitivity

Structural chromosome aberrations can be divided into two major types. Chromosome aberrations (refer to Figure 1.3) are induced following irradiation of cells in the G_0 or G_1 phase of the cell cycle, before a cell begins to undergo DNA synthesis. In this instance, the chromosome alteration produced is duplicated during DNA synthesis and can be observed when the cell is in metaphase as an aberration at the same position in both chromatids of a chromosome. Chromatid aberrations (refer to Figure 1.4) are induced in S or the G_2 phase of the cell cycle and since the cells have been exposed to radiation after the chromosomes have replicated, the chromatids can be damaged independently.

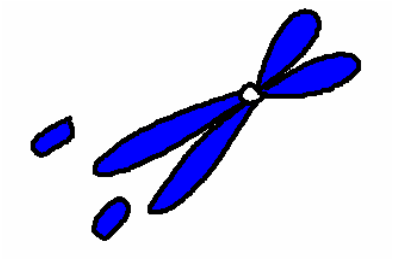
The studies performed by Sanford and colleagues at the National Cancer Institute, USA, involved X-irradiating cells *in vitro* during the G_2 phase of the cell cycle and thus chromatid type aberrations were primarily observed at the following mitosis. These aberrations can be divided into breaks, gaps and exchanges. In the work reported here, the focus was the formation of breaks and gaps, the enumeration of which provided an estimate of G_2 chromosomal radiosensitivity, as in studies described above. These aberrations reflect of a number of processes, for example, the level of damage initially produced; the amount of DNA repair that has occurred over time; or the cells ability to pass through cell cycle checkpoints to reach mitosis. As discussed above, it is defects in any of these processes that could lead to elevated levels of aberrations in assays such as the G_2 assay, enabling the discrimination of cancer prone and normal individuals.



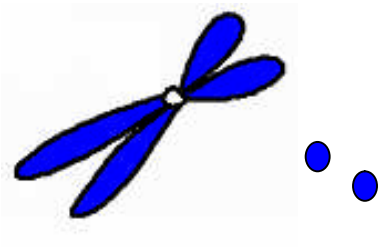
Normal chromosome



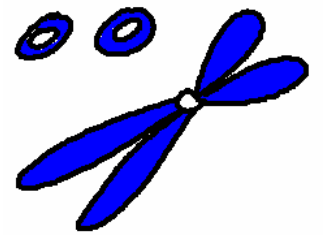
Chromosome gap



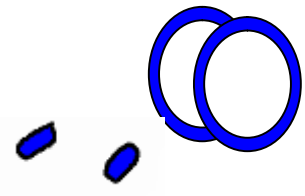
Chromosome break



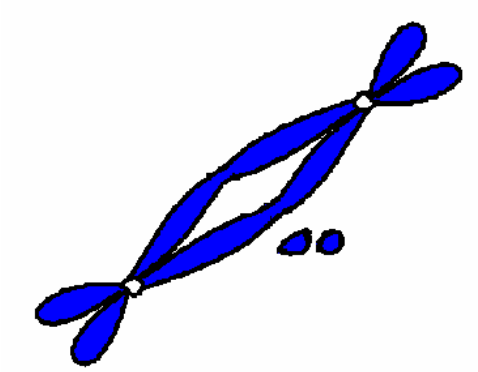
Intra-arm intrachange (minute)



Intra-arm intrachange (acentric ring)

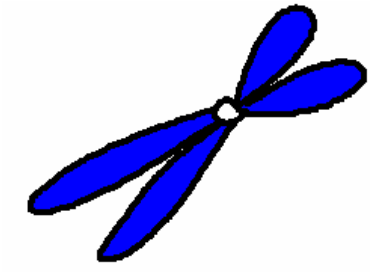


Inter-arm intrachange
(centric ring and acentric fragment)

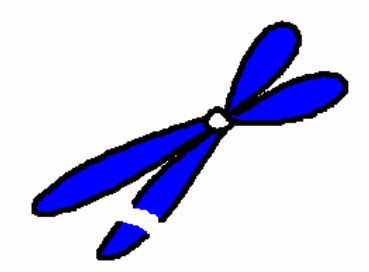


Interchange
(dicentric and acentric fragment)

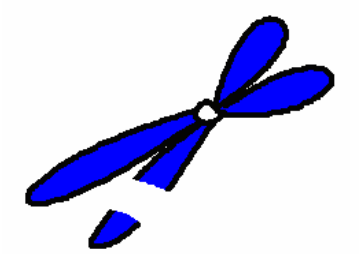
Figure 1.3: Major types of chromosome aberrations identified by solid staining.



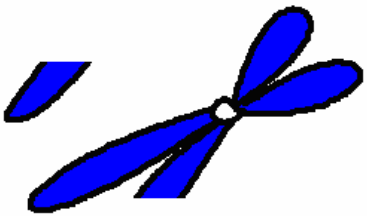
Normal Chromosome



Chromatid gap



Chromatid break
(fragment aligned)



Chromatid break
(fragment displaced)

Figure 1.4: Major types of chromatid aberrations identified by solid staining.

The mechanism underlying the formation of chromatid gaps and breaks has not yet been satisfactorily explained but traditionally two hypotheses have been used: the classical 'breakage first' model put forward by Sax (1942) and the 'exchange' model put forward by Revell (1955). Although many researchers have found that one or the other of these models provides a suitable explanation for their findings, each model is known to be unacceptable under certain conditions. The 'breakage first' (or breakage-and-reunion) model maintains that gaps and breaks are the result of single interactions of ionising tracks in chromosomes. The model maintains that following breakage of either one or both of the chromatids, rejoining of the break occurs thereby reconstituting the chromatid, or the break remains unrejoined and thus becomes visible upon condensation of the chromosomes at the subsequent metaphase. Deletion of up to a third of a chromatid would involve the loss of approximately 40 Mbp of DNA (Bryant, 1998a) and it is difficult to understand how this could occur by the passage of a single ionising track. Two explanations have been proposed. Massive exonuclease digestion from the ends of an initial DSB would have to occur in order to arrive at a 40 Mbp deletion. Such a mechanism was considered unlikely by Bryant (1998a) because it is known that mammalian cells do not significantly degrade their DNA even following high doses of radiation. Alternatively, two DSBs might be involved, acting together to cut out a large section of the chromatid. However, as Bryant (1998a) points out, at doses of 0.5 Gy (used in the G_2 experiments), the probability of two DSBs occurring in one chromatid would be extremely low. Furthermore, even if this were the case, the fragment would stay with the sister chromatid since the two chromatids are held together by cohesins and other binding proteins. Such a model would also predict a quadratic dose response curve since the probability of two DSBs occurring close together in a chromatid would increase with dose, and this is not observed experimentally (Bryant, 1998a).

The ‘exchange’ model is based on assumption that chromatid breaks are not formed at the time of irradiation but are formed later as a result of partial failure of exchanges either within or between sister chromatids (Revell, 1959). The model suggests that an exchange develops between sites of damage at the crossovers or necks of looped chromatin, and so breaks represent incomplete exchanges between undefined lesions at the point of crossing over of the loops. For this to occur, two lesions in a very small radius of crossover points of loops would be required and the chance of this is very low. Although Revell’s model doesn’t explain all the facts, it does make certain predictions for which there is good scientific evidence. The model predicts that exchanges will sometimes involve both sister chromatids, resulting in the switch of material from one chromatid to another and that if all types of chromatid breaks are equally likely then 40% of breaks would show inter-chromatid exchanges (refer to Figure 1.5). This was demonstrated in kangaroo rat cells, where it was reported that 38% of breaks showed ‘colour-switches’ when using ‘harlequin’ staining of the chromatids (Heddle *et al.*, 1969). Subsequent studies on other different species gave lower percentages (11%) of exchange associated breaks (Heddle & Bodycote, 1970). However, the fact that ‘colour-switches’ between sister chromatids at break points occur at all indicate that Revell’s model is partly correct in proposing that some form of looping and exchange occurs in the formation of chromatid breaks, and that a simple breakage-first scheme is not sufficient to explain the formation of breaks.

Bryant proposed an alternative model for the formation of chromatid breaks termed the ‘signal’ model (Bryant, 1998b). This model incorporates some elements of the Revell exchange model in that it describes events taking place in looped chromosome domains, but differs in that it accounts for the conversion of a single DSB into a chromatid break and involves signalling as a first step. In essence, the model proposes that a DSB

(occurring in a looped chromatid domain) generates a signal, which leads to misjoining of DNA ends during topoisomerase II mediated decatenation (separation of chromatids following replication) of chromatids. As a result, DNA is exchanged, inverted or excised (Bryant *et al.*, 2004). A chromatid break thus represents an incomplete exchange. Bryant (1998b) suggests the ATM protein as a possible candidate for the signalling molecule which acts by phosphorylating the histone H2AX at DSB sites following irradiation (Rogakou *et al.*, 1998). It is suggested that the 'signal' could be a physical change such as a distortion in the conformation of the loop domain, leading to errors in topoisomerase II decatenation of chromatids (Bryant *et al.*, 2004). Experiments were performed to measure the colour-switch ratio in Chinese hamster cells 2h or 4h after irradiation, when the bulk of DSB have been rejoined (Bryant, 1998b; Griffin *et al.*, 1994). About 16% of exchanges, whose incomplete forms were seen as breaks at metaphase, involved interaction between sister chromatids and were thus seen as a colour-switch at the point of breakage. However, in the majority (84%) of cases only one chromatid was involved and so a colour-switch was not observed at the break site exchange (refer to Figure 1.6). Obviously, if a recombinational exchange went to completion the break would disappear and exchanges involving both chromatids would be indistinguishable from sister chromatid exchanges.

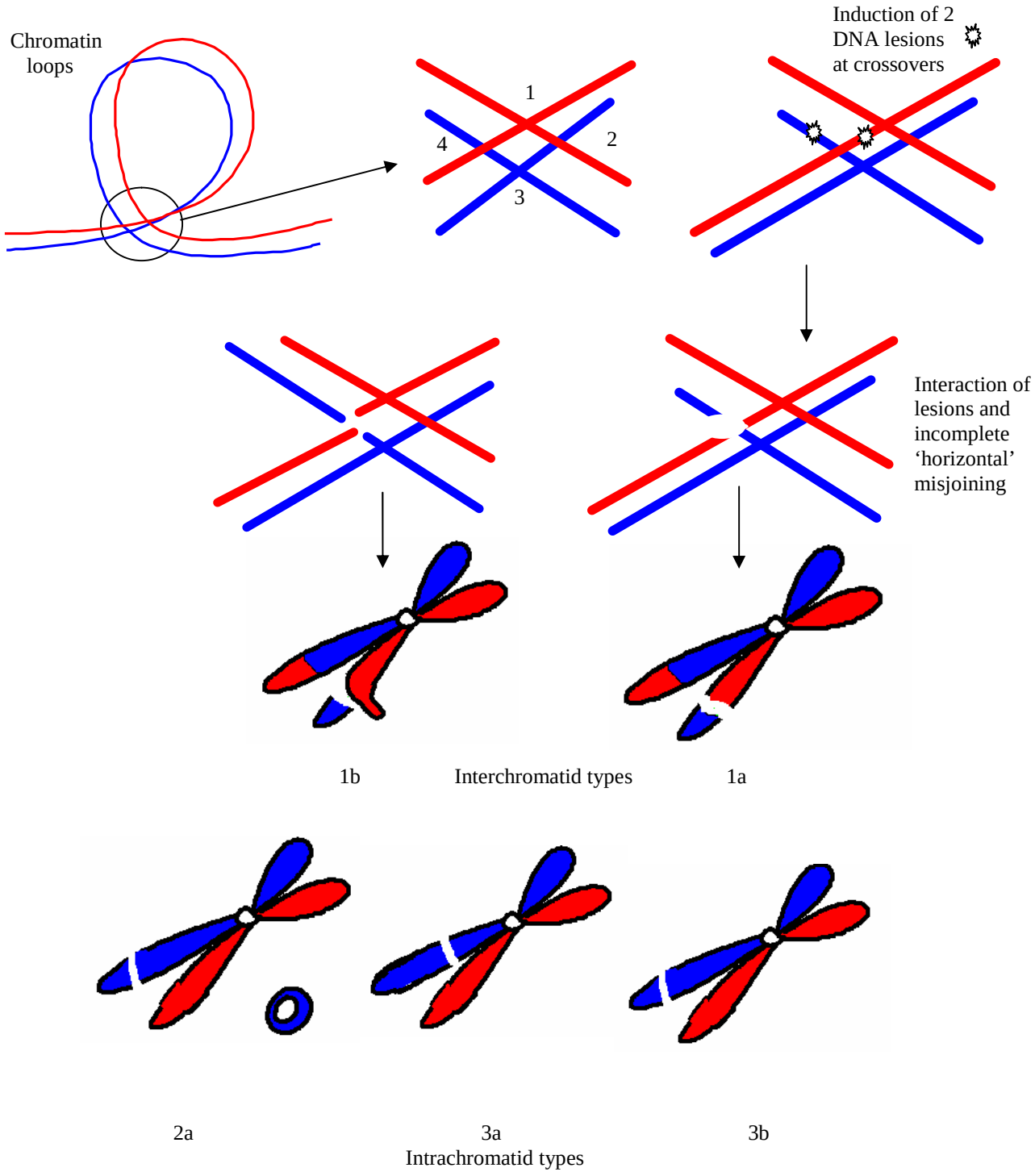
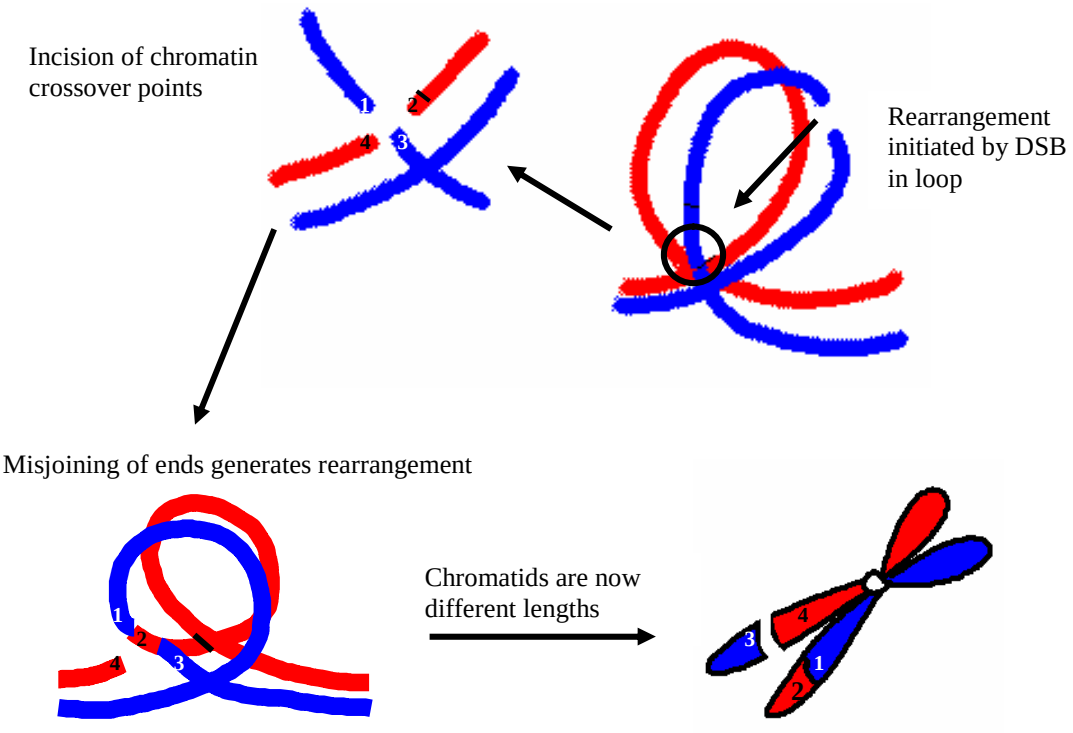


Figure 1.5: Revell's exchange model. Chromatid breaks arise by interaction of two undefined lesions occurring close together at crossover points of looped chromatin domains. Revell classified the various types of single chromatid breaks as shown.

Interchromatid break (colour-switch type)



Intrachromatid break ('omega' type)

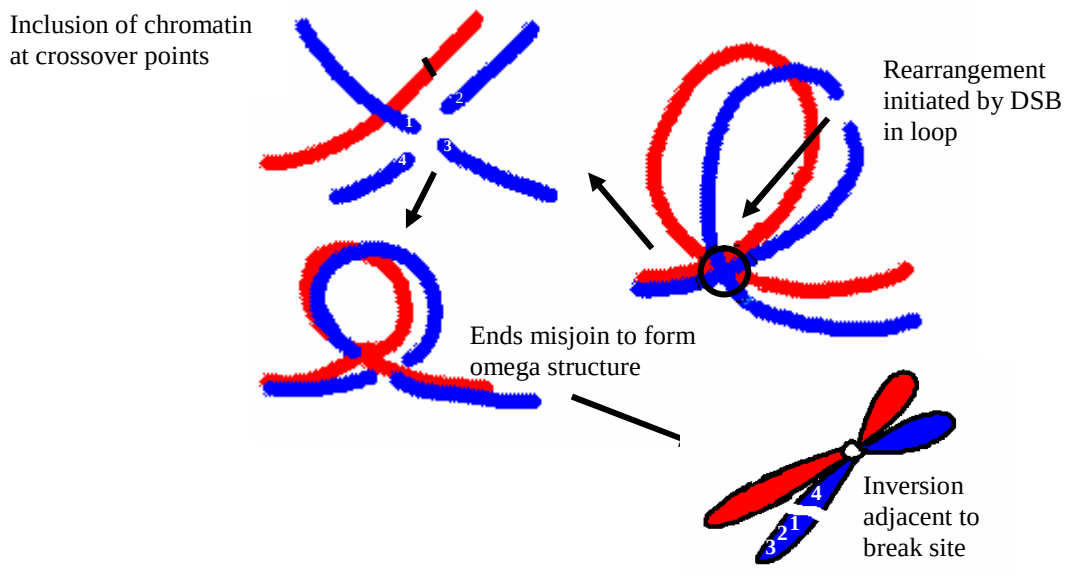


Figure 1.6: Models of possible rearrangements based on the 'signal' model leading to inter- and intra- chromatid breaks. Figure adapted from Bryant (1998b).

As stated earlier, experimental evidence suggests DNA DSBs are the most damaging lesion with respect to cell survival and they are also implicated as the principal source of chromosome and chromatid aberrations following exposure to ionising radiation. For example, X-irradiated mammalian cells treated with endonucleases that recognise and digest single strand DNA sequences thus inducing DSBs, leads to an increase in the frequency of all types of chromosome aberrations (Natarajan & Obe, 1978; Obe *et al.*, 1980). Little evidence exists to suggest that the induction rate of DNA DSBs correlate with variation in G_2 chromosomal radiosensitivity. However, despite similar initial levels of DNA damage, defects in DNA processing have been identified in cells from individuals exhibiting increased G_2 chromosomal radiosensitivity. Using the premature chromosome condensation technique, AT cells have been shown to display increased initial levels of chromatid damage compared to normal cells immediately following X-irradiation (Pandita & Hittelman, 1992a; Pandita & Hittelman, 1992b). This evidence suggests that differences in the rate of conversion of DNA DSBs into chromatid gaps and breaks could be responsible for variation observed in G_2 sensitivity (Pandita & Hittelman, 1995).

The frequency of chromatid gaps and breaks reaches maximum approximately 30 minutes post irradiation in metaphase cells. Thereafter, the frequency of aberrations falls in cells from normal individuals or remains high in cells from sufferers of cancer prone conditions (Parshad *et al.*, 1993a; Parshad *et al.*, 1983; Sanford *et al.*, 1990; Sanford *et al.*, 1987; Takai *et al.*, 1990). Evidence suggests the decline is due to DNA DSB repair. Incubating normal cells with β-cytosine arabinosidase, a polymerase inhibitor in excision repair, prevents this decline but has no effect on cells from cancer prone individuals (Parshad *et al.*, 1993b; Sanford *et al.*, 1993). This suggests cells from cancer prone individuals already

have a defect in some aspect of DNA processing involving DNA polymerase and that this could be responsible for the G_2 sensitivity observed.

As mentioned above, evidence also exists to associate defects in cell cycle checkpoints with variation in G_2 chromosomal radiosensitivity. Studies of the G_2 checkpoint in AT cells have provided somewhat confusing data. AT cells which were X-irradiated in G_1 or S-phase were observed to arrest in G_2 before they entered mitosis and this delay was prolonged compared to that of normal cells (Beamish & Lavin, 1994). However, consistent with the hypothesis that chromosomal radiosensitivity of AT cells reflects a failure of the cells to undergo the mitotic delay necessary for the repair of DNA lesions, it has been observed that AT cells irradiated in G_2 failed to arrest and proceeded to mitosis. This inverse correlation between chromosomal radiosensitivity and mitotic delay has also been shown in other cell lines (Schwartz *et al.*, 1996) and indicates *ATM* is not involved in G_2 arrest unless the damage is inflicted in the G_2 phase (Scott *et al.*, 1994b; Xu *et al.*, 2002). Indeed Xu *et al.* (2002) have shown the mechanism governing the accumulation of X-irradiated S-phase cells in G_2 is independent of the mechanisms that restrain X-irradiated G_2 cells from entering mitosis. It is clear from the experimental evidence that defects in cell cycle checkpoints play an important role in genomic instability and this may be reflected in estimates of G_2 chromosomal radiosensitivity.

The G_2 chromosomal radiosensitivity assay

The development of the cell cycle based G_2 chromosomal radiosensitivity assay was a major achievement by Katherine Sanfords group (Parshad *et al.*, 1983). The group compared chromatid damage following X-irradiation in the G_2 phase in ten lines of skin fibroblasts from individuals with genetic disorders predisposing them to a high risk of cancer with that in nine cell lines from normal adult donors. The incidence of chromatid gaps and breaks was found to be significantly higher in all the cancer prone cell lines when compared to normal cell lines except for XP-A and XP-E. Sanford interpreted the data as chromatid gaps and breaks representing unrepaired DNA damage, suggesting that cells from cancer prone individuals are deficient in some step of DNA repair (Parshad *et al.*, 1983).

The inability to confirm the experimental results of Katherine Sanfords group by others using the G_2 assay (Bender *et al.*, 1985; Bender *et al.*, 1988; Darroudi *et al.*, 1995; Scott *et al.*, 1996) highlighted a potential problem in the variability in yields of radiation induced aberrations. Indeed, several recent studies have reported significant intra-individual variation and problems with inter-individual variation (Smart *et al.*, 2003; Vral *et al.*, 2002; Vral *et al.*, 2004). One possible explanation for this could be the use of slightly different protocols by laboratories using the assay. Factors affecting the G_2 chromosomal radiosensitivity response have been identified to include pH, temperature, cell density, culture medium or serum, microbial contamination and visible light exposure (Sanford *et al.*, 1989). The assay therefore requires stringent technical conditions in order to produce meaningful and reproducible results usable as a test for detecting individuals carrying genes that increase the risk of cancer.

In an attempt to reproduce the observations of Sanford and her colleagues, Scott *et al.* (1996) applied the G_2 protocol to lymphocytes from a group of control and cancer prone individuals. Higher aberration yields, different kinetics and more inter-experiment variation than that of Sanford *et al*. was observed in control samples. Scott *et al*. put these differences down to technical features within the assay devised by Sanford *et al*., in particular centrifugation of cells prior to irradiation was found to slow down the progression of cells into metaphase and harvesting cells at 37°C was found to allow continuation of repair processes during centrifugation and hypotonic stages. From these observations, Scott *et al*. (1996) devised a modified G_2 assay protocol omitting the centrifugation step prior to irradiation and harvesting cells at 0°C. Following modification of the assay protocol in this way, Scott and his colleagues went on to confirm many of the findings of the NCI group and used the assay to study groups of sporadic cancer cases (refer to Table 1.4).

G_2 chromosomal radiosensitivity and AT

AT is a classical chromosome breakage syndrome characterised by a recessive mode of inheritance, spontaneous chromosomal instability, hypersensitivity to specific DNA damaging agents e.g. X-rays (suggesting AT cells are deficient in the ability to recognise, repair or process such damage) and susceptibility to cancer (Arlett, 1986; Taylor, 2001). The main clinical features are progressive cerebellar degeneration resulting in upper and lower limb ataxia, speech difficulties and abnormal eye movements. Initially, the complex nature of AT suggested multiple genes rather than a single gene coding for AT (Jaspers *et al.*, 1985). In 1988, a gene for AT (designated *ATM*) was localised to chromosomal region 11q22-23 by linkage analysis with DNA markers on several pedigrees (Gatti *et al.*, 1988). More recently, the AT gene has been identified by positional cloning (Savitsky *et al.*,

1995a) and has been shown to contain an open reading frame of 9168 nucleotides. The predicted protein of 3056 amino acids belongs to a family of large proteins that share sequence homologies to the catalytic domain of phosphatidylinositol-3 (PI-3) kinases (Savitsky *et al.*, 1995b). Today, research is concentrating on elucidating its molecular mechanisms and more than 100 mutations have so far been identified among AT patients. These are spread over the entire coding region of the *ATM* gene and are expected to inactivate the protein by truncation or large deletions. Heterozygous carriers of the *ATM* gene are estimated to comprise approximately 2.8% of the US white population (Swift *et al.*, 1986). They have long been a focus of interest because of two distinct features, susceptibility to cancer (especially breast cancer amongst women) and cellular radiosensitivity (Swift, 1976; Swift *et al.*, 1986; Swift *et al.*, 1987).

Many authors have investigated chromosomal radiosensitivity in AT (Bender *et al.*, 1985; Gutierrez-Enriquez *et al.*, 2004; Neubauer *et al.*, 2002; Parshad *et al.*, 1985; Shiloh *et al.*, 1989). Initial studies investigated the use of radiosensitivity, determined by clonal assays, as a laboratory based assay for carrier detection but overlap between the radiosensitivity zones of AT heterozygous and homozygous normal cells precluded their use (Arlett, 1986; Arlett & Harcourt, 1980; Arlett & Priestley, 1985; Shiloh *et al.*, 1983; Shiloh *et al.*, 1985). In 1989, Shiloh *et al*. showed that the extent of chromatid damage induced in the G_2 phase of the cell cycle by moderate doses of X-rays was markedly higher in AT heterozygous cells than in normal controls (Shiloh *et al.*, 1989). Indeed, the G_2 chromosomal radiosensitivity of many of the heterozygotes fell into the homozygote range. This enhancement in radiosensitivity was put down to the distinction made between G_2 and non-G_2 chromosomal damage. The assay was then used to diagnose the daughter of two hypersensitive parents as having AT and also confirmed the heterozygosity of the parents.

In 1990, Sanford *et al*. observed what they described as deficient DNA repair in AT heterozygous and homozygous fibroblast cells following G_2 phase X-irradiation (Sanford *et al.*, 1990). The frequencies of chromatid gaps and breaks in AT heterozygous and normal cells were not significantly different when collected during the first 30 minutes after x-irradiation suggesting the two populations of cells were equally susceptible to the radiation-induced damage. However, in metaphase cells collected between 0.5 and 1.5 hours post-irradiation, the frequencies of chromatid gaps and breaks in normal cells had declined rapidly showing a significant difference from AT heterozygous and homozygous cells. This was presumed to be due to efficient DNA repair of the radiation-induced damage in the normal cells and deficient repair during the G_2 phase in the AT heterozygotes and patients. Contrary to this, Mozdarani & Bryant (1989) showed that disappearance of chromatid gaps and breaks in AT cells and controls with time was at the same rate. From this work, Sanford *et al.* suggested that the breakdown of a system for monitoring and repairing DNA damage sustained during G_2 would lead to genetic instability, which in turn would result in the activation of oncogenes and a sequence of events that may constitute a stepwise mechanism for development of cancer (Sanford *et al.*, 1990). Thus, it was postulated that deficient DNA repair might be the defect at the molecular level that results in enhanced radiosensitivity and cancer proneness observed in AT. Studies of chromosomal radiosensitivity in AT homozygotes and heterozygotes have provided the strongest evidence for the genetic basis of an enhanced G_2 response.

G_2 chromosomal radiosensitivity and breast cancer studies

The observed association of enhanced radiosensitivity in AT heterozygotes, together with the fact that carriers of the *ATM* gene (about 1:200 in the population) have an approximate 2-7 fold increase in the relative risk of breast cancer (Easton, 1994), stimulated much

interest in the G_2 radiosensitivity profile of individuals suffering from sporadic breast cancer (Howe *et al.*, 2005; Parshad *et al.*, 1996; Patel *et al.*, 1997; Riches *et al.*, 2001; Roberts *et al.*, 2003; Roberts *et al.*, 1999; Scott *et al.*, 1999; Scott *et al.*, 1994a). In 1994, Scott *et al.* concentrated on sporadic breast cancer cases, i.e. with no obvious family history of the disease. This was mainly because most of the 5% of patients who show a clear dominantly inherited predisposition can be accounted for by mutations in either of the two identified breast cancer genes (*BRCA1 and BRCA2*). It was suggested that some of the breast cancer patients with no apparent family history could be carriers of the recessively inherited *ATM* gene. The number of breast cancer cases due to AT heterozygosity is estimated to be about 4% and possibly up to a maximum of 13%. In this instance, the G_2 assay identified 42% (21 of an unselected series of 50) of cases displaying enhanced radiosensitivity (i.e. that fell within the AT heterozygote range) compared to only 9% of controls (7/74) (Scott *et al.*, 1999; Scott *et al.*, 1994a). Scott concluded that the enhanced chromosomal radiosensitivity could be linked with a susceptibility to breast cancer and it was not confined to those with known predisposition genes or to AT heterozygotes. The proportion of breast cancer patients displaying enhanced sensitivity was much higher than 4% predicted from the population frequency of AT heterozygotes and this led to the suggestion that G_2 chromosomal radiosensitivity could be a marker of cancer susceptibility genes of low penetrance. Increased G_2 chromosomal radiosensitivity in a high proportion of cases of breast cancer has now been confirmed in several independent studies (Baeyens *et al.*, 2002; Howe *et al.*, 2005; Parshad *et al.*, 1996; Patel *et al.*, 1997; Riches *et al.*, 2001; Scott *et al.*, 1999; Terzoudi *et al.*, 2000).

If low penetrance predisposing genes do exist, as discussed earlier, then assaying for G_2 chromosomal radiosensitivity may serve as a means of identifying individuals carrying

such genes. With this in mind, other groups have assessed G_2 chromosomal radiosensitivity in individuals with other types of sporadic malignancies (Baria *et al.*, 2002; Baria *et al.*, 2001; Curwen *et al.*, 2005; Franchitto *et al.*, 2001; Papworth *et al.*, 2001; Terzoudi *et al.*, 2000)

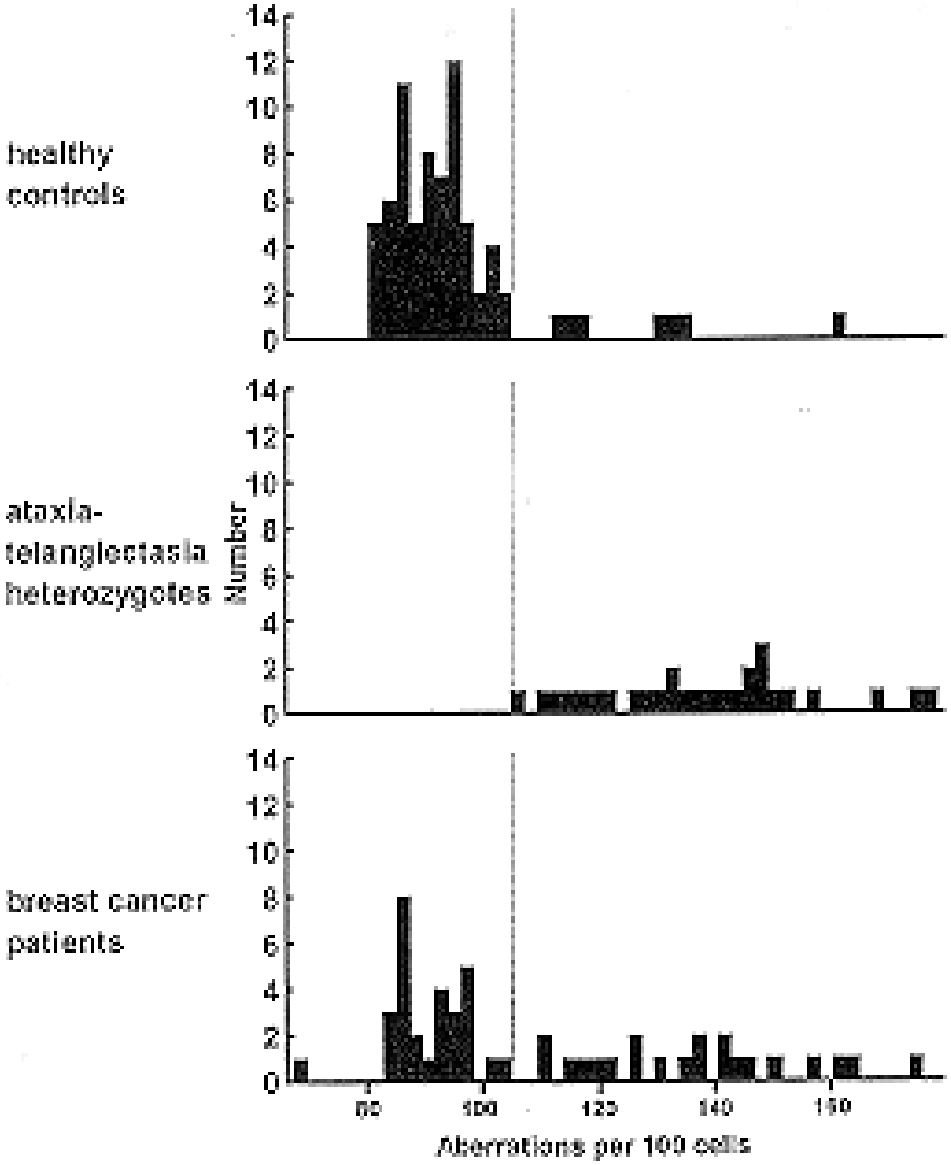


Figure 1.6: Chromosome damage in lymphocytes exposed to 0.5Gy X-rays in the G_2 phase of the cell cycle. Top panel: healthy controls (n = 74), mean aberration yield = 93.8 per 100 cells, SD = 13.6, CV = 14%; middle panel: obligate AT heterozygotes (n = 28), mean aberration yield = 145.0 per 100 cells, SD = 40.4, CV = 28%; bottom panel: breast cancer patients (n = 50), mean aberration yield = 109.0 per 100 cells, SD = 26.8, CV = 25%. Vertical line gives maximum discrimination between controls and AT heterozygotes. Figure taken from Scott et al. (1994a).

Heritability of G_2 chromosomal radiosensitivity

Further support for the hypothesis that increased G_2 chromosomal radiosensitivity may be an indicator of low penetrance cancer susceptibility genes came from the observation that blood relatives of breast cancer patients are, on average, more G_2 sensitive than normal controls (Roberts *et al.*, 1999). Of 37 first-degree relatives of 16 sensitive breast cancer patients, 23 (62%) were themselves radiosensitive compared with only 1 (7%) of 15 first-degree relatives of four breast cancer patients with normal responses (refer to Figure 1.7). The distribution of radiosensitivities among the family members showed a trimodal distribution, suggesting the presence of a limited number of major genes determining radiosensitivity. Segregation analysis of 95 blood relatives of the breast cancer patients showed clear evidence of heritability of G_2 sensitivity, and the data was reasonably well fitted by a single major gene with two alleles accounting for 82% of the variance between family members. However, since there was some evidence for a lack of fit to the single gene model and it was already known that one gene, *ATM*, confers chromosomal radiosensitivity in a proportion of breast cancer cases, a more complex genetic model was considered. A better fit was obtained from a model that included a second rarer gene, leading to the suggestion that the effect was attributable to two major genes, with similar phenotypic effects, segregating in each family.

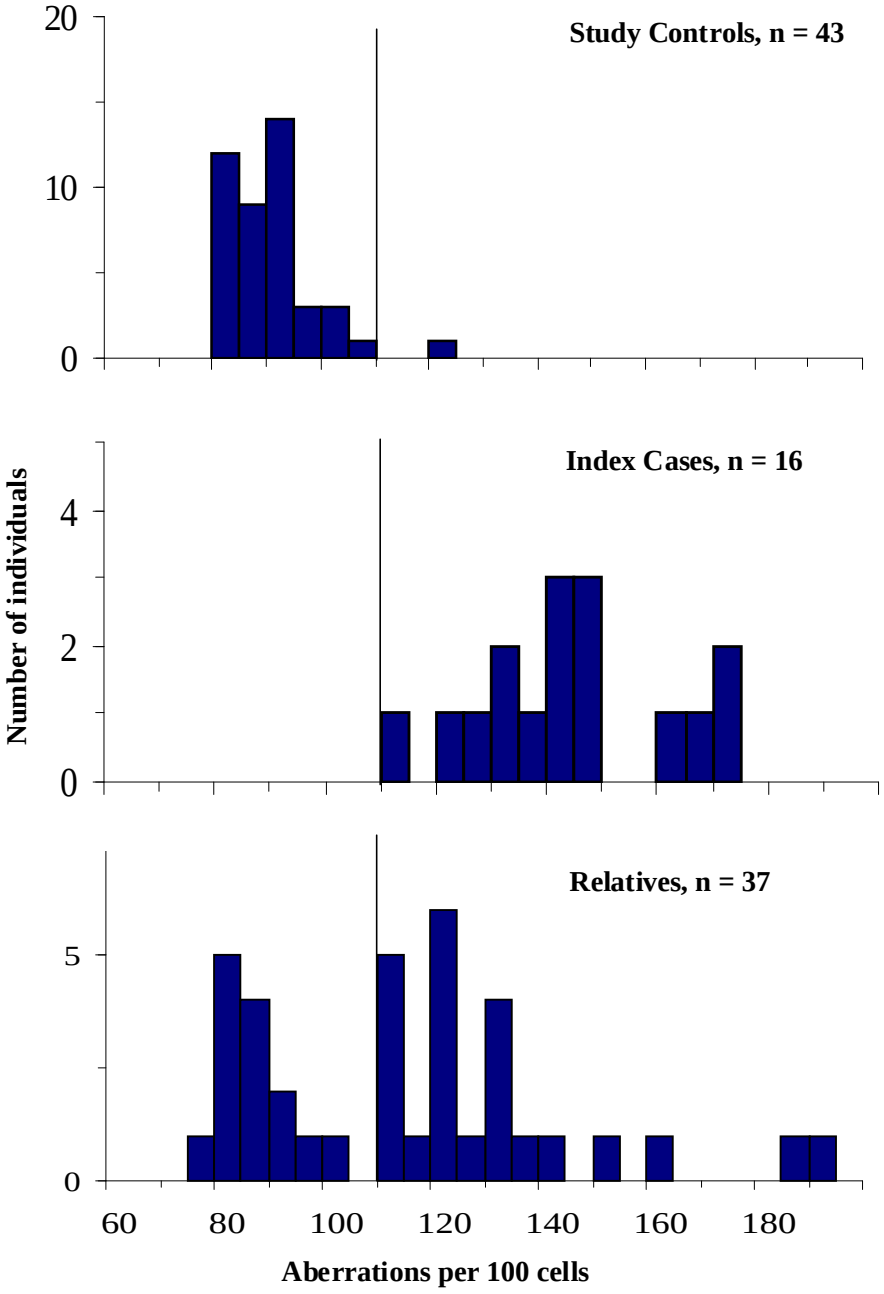


Figure 1.7: Chromosome damage in lymphocytes exposed to 0.5Gy X-rays in the G_2 phase of the cell cycle. Top panel: healthy controls; middle panel: patients with breast cancer selected as being sensitive in the assay when tested before radiotherapy; bottom panel: first-degree relatives of the patients with breast cancer who are shown in the middle panel. Figure taken from Roberts et al. (1999).

Further studies have demonstrated that the proportion of adults showing enhanced G_2 radiosensitivity is higher amongst those with cancers with an inherited component compared to those with a predominantly environmental aetiology (Baria *et al.*, 2001). Patients with colon cancer, for which there is good epidemiological evidence of an inherited risk in a substantial proportion of cases, were tested and compared to those with cancers with a predominantly environmental aetiology, such as lung cancer (linked to smoking) and cervical cancer (linked to infection with the human papilloma virus) (Baria *et al.*, 2001). Breast cancer studies were also repeated and tested alongside patients with chronic diseases other than cancer. The results showed that 30% (12/37) of colon cancer cases showed increased radiosensitivity compared with 9% (6/66) of normal healthy controls, whereas the proportions of radiosensitive cervix (11%, 3/27) and lung cancer cases (23%, 8/35) were not significantly above normals. Elevated radiosensitivity in breast cancer patients was again confirmed with 40% (12/31) of patients showing radiosensitivity. Patients with non-malignant disease showed a normal response in the assay (12% sensitive, 4/34). Baria *et al*. concluded that enhanced G_2 chromosomal radiosensitivity was a consequence of inherited defects in the ability of cells to process DNA damage and that such defects predispose to breast and colon cancer.

More recently, the G_2 assay has been applied to young patients with head and neck cancers for whom there is epidemiological evidence of an inherited predisposition in addition to environmental causes (Papworth *et al.*, 2001). A significantly greater proportion of patients less than 45 years old were more sensitive than age-matched controls, but there was no difference between patients more than 45 years old and normals. It was concluded from this work, that a greater proportion of patients with early-onset head and neck cancer are genetically predisposed compared to those with late onset disease.

In addition to the studies mentioned above, higher proportions of radiosensitive cases have been observed in a group of paediatric cancer patients compared to child controls (Baria *et al.*, 2002). Early-onset cancer is a common feature of inherited susceptibility and can be explained on the multi-stage model of carcinogenesis in that all post-zygotic cells will carry the initial genetic defect (Vogelstein & Kinzler, 1993). The G_2 assay was performed on 32 patients with early-onset cancers (diagnosed below the age of 20 years) of various types and their sensitivity compared with that of 41 young normal controls (4 months-19 years) and 32 adult normals (aged 20-60 years). The proportion of individuals showing enhanced sensitivity in each group was shown to be 44%, 15% and 10% respectively, when the 90^{th} percentile point of the adult normal group was chosen as the cut-off for enhanced sensitivity (refer to Figure 1.8). The difference between the young patients and normals was highly significant ($p = 0.004$). From the results, Baria *et al.* suggested the possibility that a substantial proportion of early-onset cancers are associated with the inheritance of susceptibility genes of low penetrance that are involved in coping with damage to the genome. It was also suggested that support for the hypothesis would require the demonstration of heritability of chromosomal radiosensitivity in blood relatives of the young cancer patients, as undertaken previously in breast cancer cases. In addition, Baria *et al.* suggested a much larger number of cases with specific, early-onset cancers should be tested to determine more accurately the proportions of sensitive malignancies of differing types.

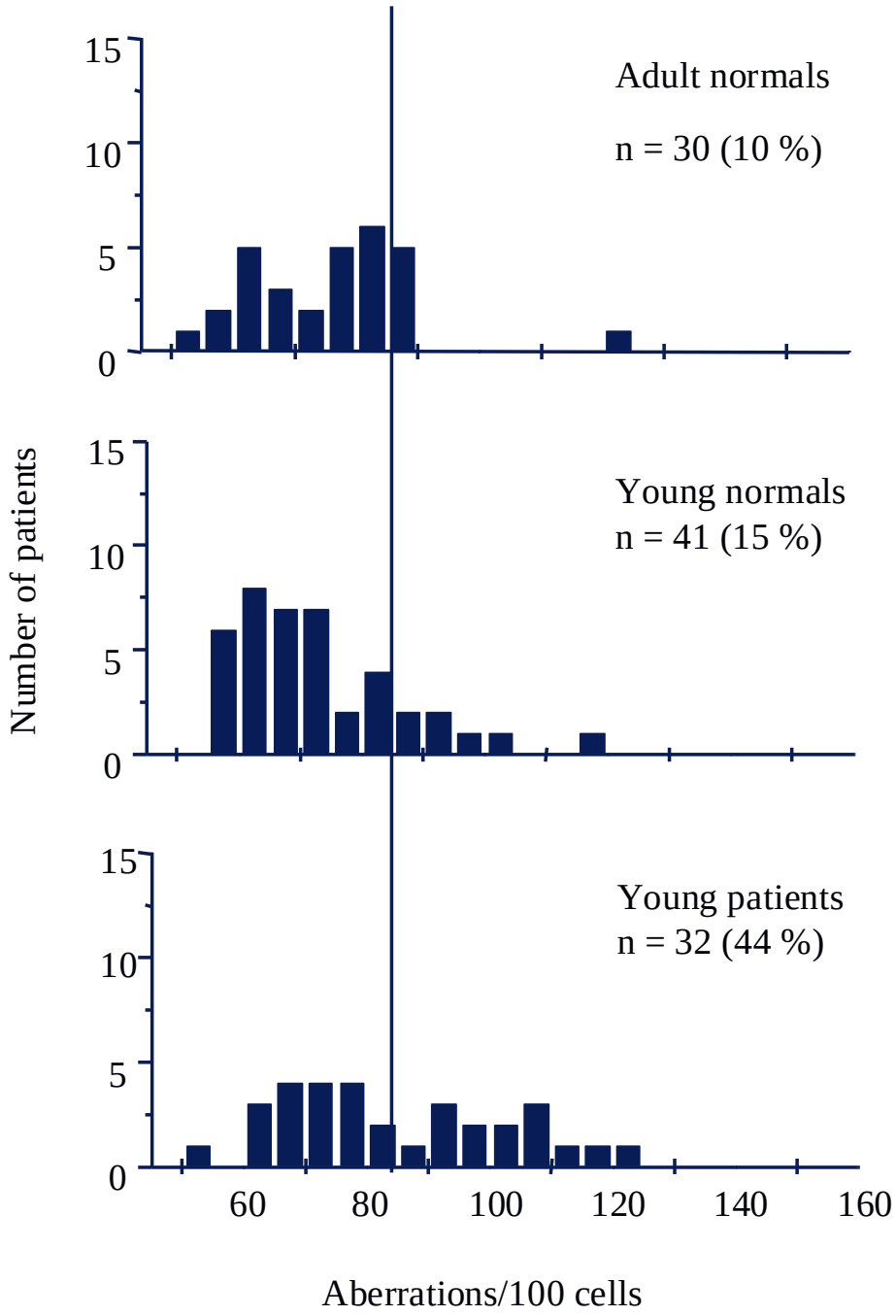


Figure 1.8: The distribution of G_2 aberration yields in three groups of donors. The solid vertical line represents the cut-off point between a normal and sensitive response as the 90^{th} percentile of adult normals. Percentages in parentheses indicate the proportions of sensitive individuals. Figure taken from Baria et al. (2002).

G_2 chromosomal radiosensitivity and polymorphic variants in DNA repair genes

Variation in radiosensitivity is influenced by genetic factors as we have undoubtedly seen with syndromes such as ataxia telangiectasia and Fanconi's anaemia. Nevertheless, such syndromes are rare and are probably of little relevance when addressing radiosensitivity among unselected cancer patients. Previous heritability studies have shown that a substantial fraction of variation in radiosensitivity is under genetic control (Roberts *et al.*, 1999). The exact genetic mechanisms underlying inter-individual differences remain unknown although control is more than likely dependent on the combined effect of several different pathways. The importance of the role of DNA repair in the protection of the genome and prevention of cancer is however appreciated (Goode *et al.*, 2002). Unravelling the human genome has revealed that single nucleotide polymorphisms (SNPs) make up 90% of the natural variation in the human genome. Previously, SNPs have been regarded as having no functional significance. However, polymorphisms located in regulatory regions may effect gene expression and SNPs causing an amino acid change alter protein function and potentially affect phenotype. Approximately 80 genes with direct roles in DNA repair and an additional 40 genes with indirect roles have been identified to date and more than 400 polymorphisms within these genes have been discovered through 'resequencing' (Mohrenweiser *et al.*, 2003). Since G_2 chromosomal radiosensitivity indirectly measures the ability of cells to repair DNA damage induced by ionising radiation exposure, variants in such genes may explain the inter-individual variation observed. Indeed, several polymorphic variants in DNA repair genes have already been reported to influence the response to *in vitro* ionising radiation susceptibility (Aka *et al.*, 2004; Au *et al.*, 2003; Godderis *et al.*, 2004; Hu *et al.*, 2001; Lunn *et al.*, 2000; Marcon *et al.*, 2003).

In this study, 13 SNPs and three microsatellite repeat regions in a total of nine genes involved in DNA repair were investigated in association with G_2 chromosomal radiosensitivity in the Danish trios. More specifically, four polymorphic genes in the BER pathway coding for XRCC1, ADPRT, APEX and hOGG1 were investigated, together with two genes, *XRCC4* and *XRCC5*, within the NHEJ pathway. In the HR pathway the genes encoding XRCC2 and XRCC3 were studied. BER and HR are important pathways in repairing damage by ionising radiation, repairing oxidative base damage and DNA strand breaks. Thus, defective or deficient repair activities may contribute to increased sensitivity to ionising radiation and elevated cancer risk. Many SNPs have been identified that are specifically involved in BER and HR pathways and those with functional significance could potentially be classified as cancer susceptibility genes. Low penetrance susceptibility alleles may also contribute to many cancer cases since some are very common in the general population.

XRCC1 has an important role in BER by acting as a scaffolding intermediate interacting with ligase III, DNA polymerase III, and ADPRT (poly(ADP-ribose)polymerase-1 or also known as PARP) in the C-terminal, N-terminal and central regions of the protein, respectively (Hu *et al.*, 2001). XRCC1 interacts with ADPRT to form a complex which binds to, and is activated by, single strand breaks (Hoeijmakers, 2001). *XRCC1* mutants have increased sensitivity to ionising radiation, ultra violet, hydrogen peroxide and mitomycin C (Thompson & West, 2000). Two variant alleles (g1301a and c685t) have been associated with an increased risk for oral and pharyngeal cancers. The variant allele g1301a results in a non-conservative substitution in a hydrophobic region of XRCC1, whilst the c685t SNP occurs within BRCA1 C-terminal domain known to interact with PARP and has also been associated with lung cancer risk. APEX is a rate limiting enzyme

in the BER pathway, involved in the excision of abasic sugar residues generated from exogenous factors such as ionising radiation and environmental carcinogens (Hoeijmakers, 2001) which are then repaired by an XRCC1/DNA polymerase β complex.

Poly(ADP-ribosyl)ation is a eukaryotic cells response to aid cellular recovery from oxidative and other types of DNA damage. The process involves post-translational modification of nuclear proteins and is catalysed by the nuclear enzyme poly(ADP-ribose)polymerase-1 (PARP) with NAD+ serving as the substrate. The amino terminal of PARP binds to SSBs or DSBs breaks in DNA causing activation of the catalytic centre in carboxy-terminal of the NAD+ binding domain. In addition to a role in BER, poly(ADP-ribosyl)ation has been proposed to be involved in DNA replication, gene transcription, cell differentiation (Cottet *et al.*, 2000).

Reactive oxygen species formed continuously in living cells can result in DNA damage such as mutations which can result in the activation of oncogenes or inactivation of tumour suppressor genes and thus eventually cancer. 8-hydroxyguanine (8-oxoG) is a major form of DNA damage produced by reactive oxygen species and causes G:C to T:A transversions which are widely seen in tumours. The DNA repair enzyme hOGG1 is specifically involved in catalysing the excision of 8-oxoG lesions (Wikman *et al.*, 2000). Several variant *hOGG1* alleles have been identified, with two variants (c1285g and g445a) shown to have weaker 8-oxoG repair capacity. Other variants have not been studied widely.

XRCC4 forms a complex with DNA ligase IV which binds to, and links, the ends of DNA whilst XRCC5 (Ku 86) contributes to NHEJ as part of the Ku p70/p86 dimer which acts to stabilise and bring together broken DNA ends (Mohrenweiser *et al.*, 2003). *XRCC2* and

XRCC3 are members of the *RAD51* DNA repair gene family and function in the HR pathway by complexing with RAD51c and RAD51b in DSBs and cross-link repair. *XRCC2* and *XRCC3* mutant cells show moderate hypersensitivity to ionising radiation and extreme sensitivity to DNA cross-linking drugs such as mitomycin C.

XPA to XPG are genetic complementation groups encoding proteins involved in the NER pathway. NER is composed of two sub-pathways: global genome repair which repairs damage to inactive genes and transcription coupled repair which repairs damage to the transcribed strand of active genes. XP results from defects in both mechanisms linked via the TFIIH complex (a multi-protein basal transcription factor that participates in NER and transcription initiation). XPB and XPD are components of TFIIH and thus interact with many different proteins, explaining their involvement in diseases with different phenotypes. The XPD protein has both single strand DNA-dependent ATPase and 5'-3' DNA helicase activities and is thought to participate in DNA unwinding during NER and transcription. XP mutant cell lines are deficient in ionising radiation repair, which is preferentially repaired on the transcribed strand suggesting a role for the XPD protein. Since XPD has an obvious importance in multiple cellular tasks and the variant *XPD* allele a2251c has previously been reported to influence G_2 chromosomal radiosensitivity (Lunn *et al.*, 2000), polymorphic variation of the *XPD* gene was also examined.

CHAPTER 2

The G_2 chromosomal radiosensitivity assay validation study

Introduction

An initial validation study was undertaken on a group of healthy control volunteers to ensure the G_2 assay was fully standardised in the WRI laboratory and that reproducible results could be demonstrated before the Danish trio study (refer to Chapter 3) commenced. The effects of storage and culture conditions were examined and the extent of intra and inter-individual variations determined.

Materials and Methods

Validation study population

Initially, peripheral blood samples were collected from 19 adult WRI staff although this was later increased to 27 to be more comparable with the number of Danish families collected (refer to Chapter 3). Ethical approval for the study was sought from the North Cumbria Local Research Ethics Committee (LREC). Volunteers gave informed consent and samples were coded to ensure anonymity. Donors included 11 males and 16 females, age range 21 to 54 (refer to Table 2.1).

Sample collection

Samples were collected from the first group of 19 donors between March 2001 and January 2002 and the additional eight were collected over two weeks in June/July 2004.

All blood samples were drawn into 5ml lithium heparin vacutainers (BD Vacutainer Systems, Ref. 367684) by the WRI research nurse based at the West Cumberland Hospital, Whitehaven, and transported back to the WRI, a distance of approximately 2km, by car. During transit, vacutainers were stored at ambient temperature in screw-cap polyethylene tubing packed with absorbent cotton wool to avoid breakage and absorb any possible spillage.

Dose response experiments

An initial set of experiments was performed on a single blood sample provided by one normal volunteer to construct a dose response curve for irradiation. This was to investigate the effect of different doses on the induction of chromatid aberrations in human lymphocytes in the G_2 phase of the cell cycle at the time of irradiation and also for comparison to dose response data obtained at different laboratories. The blood sample was split into five fractions and the G_2 assay performed on duplicate cultures for each fraction, exposing each pair to different doses of X-rays (0, 0.25, 0.4, 0.5, 0.6 Gy). Chi-squared analysis revealed no statistically significant difference between either culture set up for each dose (refer to Table 2.2) and thus mean induced chromatid aberration frequencies per 100 cells were used when constructing the response curve (refer to Figure 2.1). The relationship between induction of aberrations and radiation dose was found to be linear. A dose of 0.5 Gy was chosen for irradiations in all subsequent experiments.

Assay reproducibility, blood storage and transportation

In order to determine assay reproducibility, four replicate cultures were set up using blood from a single sample donated by donor N and the G_2 assay performed on each (refer to Table 2.3). Since it was not always possible to set blood cultures up for the G_2 assay

immediately after they had been drawn, assay reproducibility following extended blood storage was also assessed. This was performed by undertaking the G_2 assay on an additional four cultures set up from the same blood sample used to determine assay reproducibility that had been stored at room temperature for 24 hours. The effect of delaying culturing for 24 hours was investigated further, in a similar manner as above, using additional blood samples obtained from donors N and O. Cultures were set up immediately for the G_2 assay using blood from a single sample and again, following overnight storage of the remaining blood at room temperature. Table 2.3 provides details of the samples received, how they were split and how many cultures were set up. In addition, the effect of temperature on blood storage was investigated by splitting the same samples obtained from donors N and O further and storing them overnight at 4°C in the refrigerator.

The effect of transporting blood samples was investigated by splitting samples received from donors D, O, K and Q into two and setting up blood cultures for the G_2 assay immediately with one half of the sample, whilst the other half was transported in secondary polyethylene tubing and carried inside a metal sample carrier box in the boot of a car, from early morning (approximately 9 am) to early evening (approximately 6 pm) and transported a distance of 15 miles before being stored overnight at room temperature and then being transported in the same manner, another 15 miles at approximately 7 am the following morning, back to WRI where blood cultures were set up. Table 2.3 provides sample details.

Intra- and inter-individual variations

A single blood sample was collected from 18 of the donors whilst for the remaining nine,

at least 3 and up to 13 repeat samples were tested, giving a total of 65 samples. Intra-individual variation was assessed on the individuals who provided repeat samples whilst inter-individual variation was assessed from the results of all 27 volunteers.

Table 2.1: Characteristics of validation study donors and number of samples provided.

Donor	A	B	C	D	F	H	I	J	K	L	M	N	O	P	Q	T	U	V	W	X	Y	Z	CC	DD	EE	FF	GG
Sex	F	F	F	M	M	F	M	F	F	M	F	F	F	F	F	M	M	M	M	M	F	M	F	M	F	F	F
Age[1]	28	30	27	47	29	27	33	26	34	48	54	36	48	21	44	20	29	22	22	54	33	48	23	42	21	36	21
Samples[2]	3	1	1	5	3	1	1	3	7	1	1	13	7	3	3	1	1	1	1	1	1	1	1	1	1	1	1

[1] Age at time of sampling, [2] Number of samples provided.

Table 2.2: Chromatid aberration frequencies with increasing dose.

Dose (Gy)	Chromatid aberration frequency per 50 cells		Mean ± sd[1]	CV[2] (%)	χ^2	*P*
	Culture 1	Culture 2				
0.25	26	22	24.00 ± 2.83	11.79	0.22	0.640
0.40	22	31	26.50 ± 6.36	24.01	1.01	0.316
0.50	37	47	42.00 ± 7.07	16.84	0.78	0.376
0.60	58	75	66.50 ± 12.02	18.08	1.43	0.232

[1] Standard deviation, [2] Coefficient of variation.

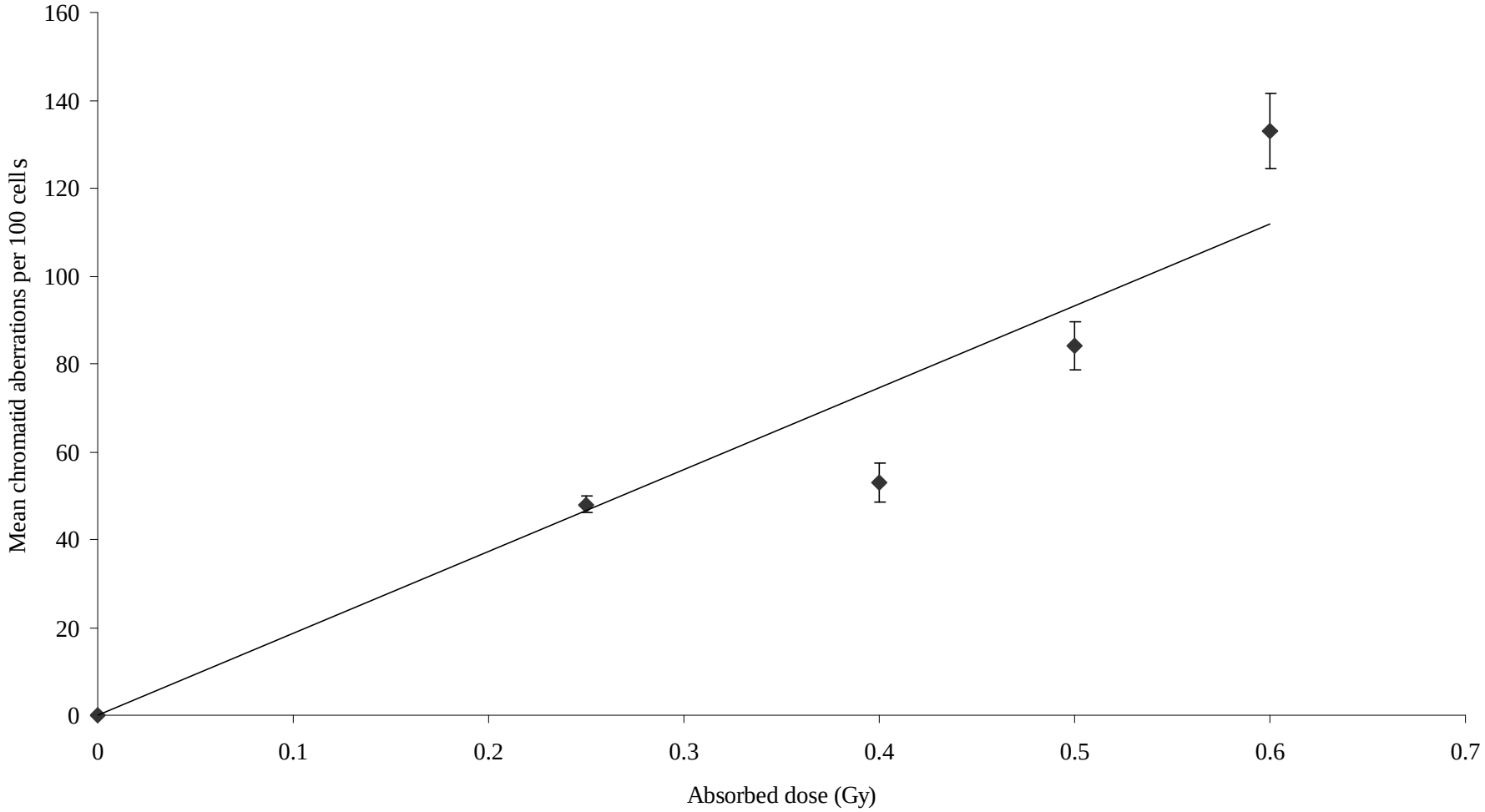


Figure 2.1: The frequency of chromatid aberrations with increasing dose.

Table 2.3: Details of validation samples collected to test assay reproducibility, blood storage and transportation conditions.

For each variable tested, details are provided on both test samples and corresponding controls.

Donor D	Assay Reproducibility	Storage at RT for 24hrs	Storage at 4°C for 24 hrs	Transportation
				Sample 4a immediate culture Sample 4b travelled (collected 14/05/01)
N	Sample 9a-d (4 cultures) Sample 9e–h (4 cultures) (collected 23/07/01)	Sample 8a immediate culture Sample 8b RT for 24 hrs (collected 16/07/01) Sample 9a-d immediate culture Sample 9e-h RT for 24 hrs (collected 23/07/01) Sample 12a & 12b immediate culture Sample 12c & 12d RT for 24 hrs (collected 03/12/01)	Sample 11a immediate culture Sample 11b 4°C for 24 hour (collected 08/10/01) Sample 12e & 12f 4°C for 24 hour (collected 03/12/01)	
O		Sample 6a & 6b immediate culture Sample 6c RT for 24 hour (collected 08/10/01) Sample 7a & 7b immediate culture Samples 7c & 7d RT for 24 hrs (collected 03/12/01)	Sample 6d & 6e 4°C for 24 hour (collected 08/10/01) Samples 7e & 7f 4°C for 24 hour (collected 03/12/01)	Sample 4a immediate culture Sample 4b travelled (collected 14/05/01) Sample 5a immediate culture Sample 5b travelled (collected 21/05/01)
K				Sample 5a immediate culture Sample 5b travelled (collected 14/05/01) Sample 6a immediate culture Sample 6b travelled (collected 21/05/01)
Q				Sample 2a immediate culture Sample 2b travelled (collected 14/05/01) Sample 3a immediate culture Sample 3b travelled (collected 21/05/01)

a,b,c,d,e,f,g,h = fractions a single blood sample was split into.

Determining G_2 chromosomal radiosensitivity

The 'G_2 assay' was identified as the most sensitive assay to examine chromosomal radiosensitivity in the sample populations investigated as cell cycle based assays are thought to provide the greatest power for discriminating differences in response to DNA damage. Essentially, the G_2 assay registers chromatid damage occurring during the G_2 phase of the cell cycle and is manifest as chromatid gaps and breaks at the subsequent metaphase. The assay was also the method of choice in several previous studies of a similar nature (Baria *et al.*, 2002; Papworth *et al.*, 2001; Riches *et al.*, 2001; Scott *et al.*, 1996; Scott *et al.*, 1994b).

The G_2 assay

Unless otherwise stated, samples were cultured within one hour of blood being drawn and two cultures were set up for the G_2 assay. One blood culture was then irradiated and the other acted as a non-irradiated control to enable the determination of the spontaneous chromatid aberration yield. The G_2 assay was performed according to a modified version of the method of Scott *et al.* (1999), which was originally based on and developed from the work of Sanford *et al.* (1989). Twenty ml cultures containing 2ml whole blood and 18ml pre-warmed (37°C) and pre-gassed (5% CO_2/95% air) RPMI-1640 medium (Sigma, Ref. R8758) supplemented with 15% foetal calf serum (GibcoBRL, Ref. 10099, Batch no. 30R0014S), 1% 200mM L-Glutamine (Gibco, Ref. 25030-032) and 1% PHA M-form (Gibco, Ref. 10576-015), were set up in 25cm^3 tissue culture flasks (VWR Int., Ref. 402/0446/02). Complete RPMI-1640 medium was prepared by removing 17mls medium from a 100ml bottle and adding 15ml of foetal calf serum and 1ml each of L-Glutamine and PHA. PHA is a mitogen that acts by stimulating lymphocytes to become transformed into DNA-synthesising and mitotically active cells (Chandler & Yunis, 1978). It causes an

increase in RNA synthesis in cells *in vitro* after a culture time lag of 24 hours and then during the next 24 hours, the nucleus enlarges and DNA synthesis begins. The first mitoses are seen at around 48 hours, with waves at 24 hourly intervals thereafter. Flasks were placed upright in a 5% CO_2 gassed incubator at 37°C for 72 hours. The medium is supplemented with foetal calf serum and L-Glutamine (a non-essential amino acid) to provide the cell culture with factors identified as essential for growth such as growth and adhesion factors, mineral trace elements, hormones, binding proteins and vitamins.

After 48 hours of culture, 15ml of spent medium was carefully removed from above the cell layer and replaced with freshly pre-gassed and pre-warmed media. After 72 hours culturing, flasks were transported to the X-ray machine (Seifert Isovolt 320 kV Radiographic X-ray System) in a 37°C portable incubator where they were irradiated (or mock irradiated for control samples) with 0.5 Gy of 300kV X-rays and then returned to the main incubator. Following a 30 minute recovery period, cultures were treated with 0.2ml Karyo MAX colcemid (10µg/ml) (Gibco, Ref. 15210-057). Colcemid acts as a mitotic arresting agent introducing a block between metaphase and anaphase by preventing spindle formation and thus allowing the production of metaphase chromosome spreads which are compacted and visible under the microscope. Following a 1 hour incubation with colcemid, the contents of the culture flasks were transferred to centrifuge tubes and at exactly 90 minutes after irradiation, plunged into ice chippings to rapidly cool so inhibiting further DNA repair. All subsequent steps were carried out at 4°C. Following centrifugation at 1500rpm for 5 minutes, the supernatant was removed from above the cell layer and the remaining pellet vortexed to resuspend cells. Ice-cold hypotonic solution (prepared by dissolving 5.595g potassium chloride (VWR Int., Ref. 101984L) in one litre of distilled water to produce a 0.075M solution) was added to the cells by filling centrifuge

tubes to the 10ml mark and mixing to invert. Treatment with hypotonic for 20 minutes causes lymphocytes to swell and red blood cells to burst. During this time centrifuge tubes were inverted to mix the contents every 5 minutes. Following hypotonic treatment, tubes were centrifuged at 1500rpm for a further 5 minutes, supernatant removed and cells fixed by the dropwise addition of 10ml freshly prepared fixative consisting of three parts methanol (VWR Int., Ref. 10158-6B) and one part acetic acid (VWR Int., Ref. 10001CU). Fixed cells were washed a further six times by centrifuging the tubes at 1500rpm for 5 minutes, each time removing the supernatant from above the cell layer and vortexing to resuspend the pellet, then adding 10ml freshly prepared fixative and inverting to mix. Tubes containing fixed cells were stored at –20°C overnight or for longer. Figure 2.2 provides an illustration of sample collection and the G_2 assay method employed.

Slide preparation

Only cell suspensions that had been washed in fixative at least six times, so ensuring a clean cell preparation, were used for metaphase slide preparation using standard procedures (Rooney, 2001). Since irradiation results in a reduced mitotic index, it was necessary to make six slides to obtain enough metaphases to score for each irradiated sample but only two slides were necessary for each non-irradiated control. Slides (Merck, Ref. 406/0169/02) were cleaned by storage in a tank of methanol (VWR Int., Ref. 10158-6B) at least overnight and, prior to usage, rinsed in distilled H_20 to remove the methanol and polished using clean white tissues (Fisher Scientific, Ref. CMC-650-032Q). Slides were then immersed in ice chippings for at least 30 minutes to cool and wet in order to aid chromosome spreading.

Chromosome spreading and staining

Cell suspensions were removed from the freezer and allowed to equilibrate to room temperature for 30 minutes before being centrifuged at 1500rpm for 5 minutes and the supernatant aspirated to approximately 1cm above the cell pellet. The remaining pellet was vortexed to mix, washed once in 10ml fresh fixative, centrifuged at 1500rpm for 5 minutes and the supernatant aspirated to just above the cell pellet again. Cell pellets were resuspended in approximately 0.5ml fresh fixative to make a milky suspension and using a Gilson pipette, 40µl of the suspension was dropped from a height of approximately 50cm into the middle of a cold, wet slide that had been removed from the ice bucket and had excess ice and water blotted from it. To further aid chromosome spreading, slides were edge flamed which involved passing the slide quickly through the top of a flame, then allowed to air dry.

Slides were arranged back to back in a trough and stained with 5% v/v R66 Giemsa (Merck, Ref. 350864X) in Gurrs buffer, then rinsed twice with Gurrs buffer and left to air dry. Gurrs buffer was prepared by dissolving one tablet (BDH Chemicals Ltd., Ref. 33199) into one litre of distilled water producing a solution of approximately pH6.8 at 20°C. Finally, slides were mounted with 22x50mm coverslips (Merck, Ref. 406/0188/42) using DPX mountant (VWR Int., Ref. 360294H) to reduce fading and ensure quality. Following analysis, fixed cell suspensions were transferred to 1.5ml NUNC cryotubes (Scientific Laboratory Supplies, Ref. 375418K), labelled with the sample ID number and stored at -20^{o}C in polycarbonate cryoboxes (Ross Lab, Ref. B26494). The laboratory number, freezer location and box number was recorded on a 'Fixed Cells Storage Location Sheet' for future reference (refer to Appendix A).

Scoring criteria

All slides were coded for anonymity and scored blind. Slides were placed on the microscope stage with the coloured/frosted edge on the right hand side. Initially, slides were scanned at x10 magnification from one corner working up and down the slide until a metaphase was found. Only well spread metaphases were selected. The magnification of the microscope was changed to x100 using oil immersion and the quality of the metaphase assessed again before scoring for any chromosome damage. Chromosome pieces were counted and if there were less than 46, the cell discarded. If there were 46 or more chromosome pieces, the number was recorded in the appropriate column on the 'G_2 Radiosensitivity Score Sheet' (refer to Appendix B) along with the microscope verniers, so that the cell could be easily relocated. Chromosomes were also checked for the presence of centromeres and recorded as normal if one was present in each chromosome. Cells in metaphase of the cell cycle were then analysed for chromatid aberrations. These were recorded on the 'G_2 Radiosensitivity Score Sheet' and were classified according to Sanford *et al*. (1989) and Scott *et al*. (1999) who defined breaks as chromatid discontinuities with displacement of the broken segment and gaps as showing no displacement of the segment distal to the lesion. Figure 2.3 illustrates the chromatid aberrations observed. Gaps were only included in the final G_2 score if wider than the chromatid width. Smaller lesions were scored but not included in the final results. Chromatid gaps and breaks were added together to give the total G_2 score for each irradiated and non-irradiated control sample and the induced and spontaneous chromatid aberration yields determined. Two scorers, each scoring 50 metaphases per irradiated and non-irradiated control sample. Both aberration scorers were checked periodically throughout the study by a third independent scorer, to ensure the set scoring criteria was consistent. This was achieved by scoring the same 50 cells and comparing results.

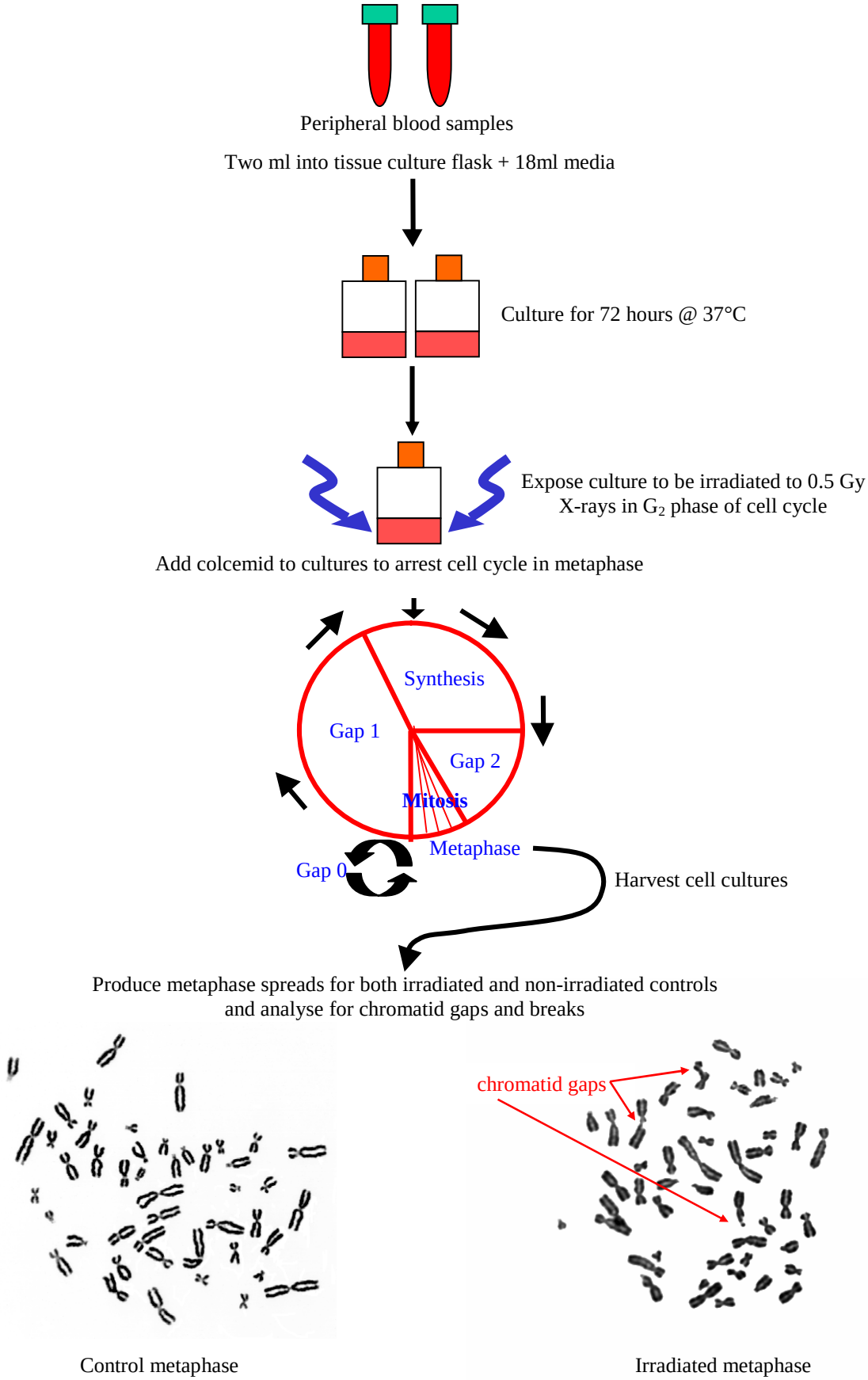


Figure 2.2: Sample collection and the G_2 assay procedure.

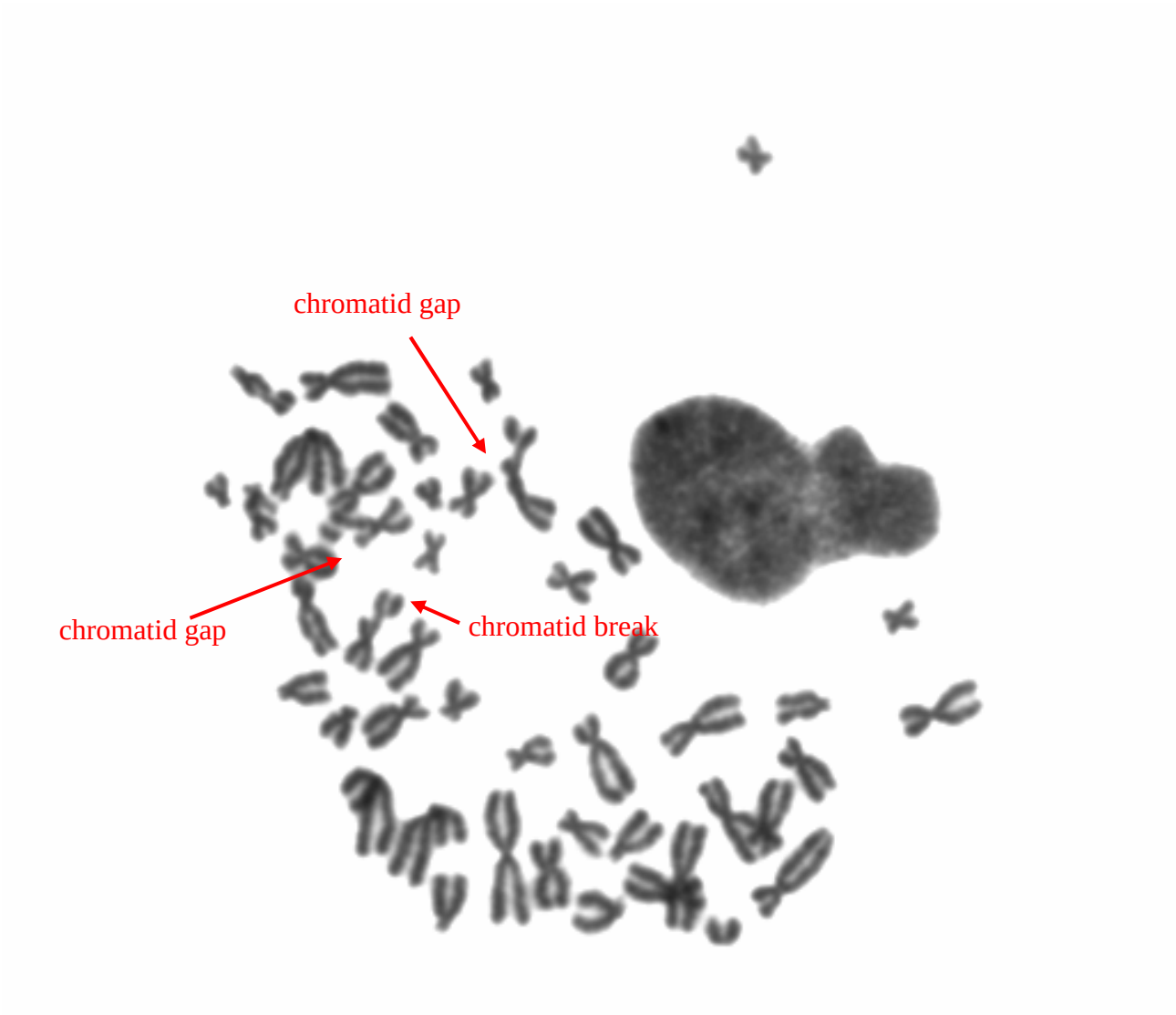


Figure 2.3: Irradiated metaphase illustrating 46 Giemsa stained chromosomes displaying chromatid gaps and breaks.

Statistical analysis

Analysis of aberration distribution

Poisson statistics were used to study the distribution of chromatid-type aberrations amongst metaphase cells as previously described by Savage (1970). This distribution is used to model data that are counts of (random) events in a certain area or time interval, without a fixed upper limit. The chance of a cell developing an aberration is randomly distributed i.e. every cell has an equal chance, if the observed distributions follow Poisson statistics. In mathematical terms, this is represented by the variance and the mean of the observed distributions being equal and a ratio value of 1.0 would be expected. If the variance is greater than the mean, aberrations are overdispersed and a Poisson distribution does not apply. Ratios of variance to mean (the mean being the number of aberrations divided by the number of metaphase cells studied) for each sample provided by a donor were calculated using Microsoft Excel and the average value determined.

Estimating intra- and inter-individual variability

Chi-squared (χ^2) analysis was used to investigate homogeneity of repeat sampling in the in those donors who provided repeat samples. Chi-squared adopts the formula $\chi^2 = \Sigma(O–E)^2/E$, where O is the observed value of aberrations per 100 cells and E is the expected value of total aberrations per 100 cells. Overdispersion of distributions of aberrations amongst metaphase cells was corrected by multiplying the expected value of total aberrations per 100 cells (E) in the denominator of the equation for chi-squared by a compensation factor (Z = average value of ratio of variance to mean) and so the formula $\chi^2 = \Sigma(O - E)^2/(EZ)$ was adopted. Chi-squared analysis was again used to assess variation between individuals with overdispersion taken into account as above. Since multiple samples were collected from some but not all individuals, additional intra-individual

variation observed was also compensated for. This was estimated by adding all the values of chi-squared for those individuals who were sampled more than once and dividing by the total degrees of freedom.

Mean and median induced aberration yields

Mean induced aberration frequencies were calculated by dividing the total number of aberrations observed by the total number of cells scored and normalising to 100 cells scored. Repeat sampling was undertaken on nine individuals (refer to Table 2.4) and for the calculation of mean induced aberration frequency, individual means from these donors were used together with single sample results of the remaining 18 samples.

Standard errors

Standard errors were calculated by adjusting for overdispersion of chromatid-type aberrations using the appropriate compensation factor (Z = average value of ratio of variance to mean). In addition, where repeat sampling had occurred, any additional intra-individual variation introduced was also compensated for. This was achieved by calculating all the values of chi-squared for those individuals who were sampled more than once and dividing by the total degrees of freedom and is termed Y. Standard errors were thus calculated according to the formula $\sqrt{}$(Number of aberrations x Z x Y), and normalised to 100 cells scored.

Determining radiosensitivity

The proportion of radiosensitive individuals was determined by using a cut-off value of the 90^{th} percentile of chromatid aberration frequencies per 100 cells, as suggested by Scott (Roberts *et al.*, 1999; Scott *et al.*, 1999).

Results

Results were obtained from 27 WRI staff who donated blood for the validation study, giving a total of 65 separate samples. Chromatid aberration yields (number of gaps and breaks per 100 metaphases) at the different sampling points and the mean induced chromatid aberration frequency ± S.E. for each donor are given in Table 2.4. Analysis of chromatid aberration distributions amongst the cells revealed an overdispersion compared to that predicted by random dispersion (Poisson distribution), with an average ratio of variance to mean of 1.52. Overdispersion was taken into account by adjusting the expected values for the yields of chromatid aberrations per 100 metaphases analysed in subsequent equations, by a factor of 1.52. The mean induced chromatid aberration yield ± S.E. determined from all 65 samples was 95.06 ± 2.05 per 100 cells (range 63-155). In comparison, using individual mean induced chromatid aberration frequencies from those donors sampled more than once, together with single sample results for the remaining 18, resulted in a mean induced chromatid aberration yield ± S.E of 100.91 ± 3.28.

Intra- and inter-individual variations

Single blood samples were collected from 18 of the validation study donors, whilst the remaining nine were sampled repeatedly (range 3–13 samples). Intra-individual variation was determined from those donors sampled on more than one occasion and the results are given in Table 2.4. Seven out of nine individuals displayed an intra-individual variation that was not statistically significant. Donor F was of borderline significance at the 95% confidence limit (χ^2_2 = 6.03, P = 0.049) and this was mainly due to a high chromatid aberration frequency in one of the samples collected from that individual. Removal of this sample resulted in an intra-individual variation that was not statistically significant (mean

= 100.50 ± 12.01, χ^2_1 = 0.95, *P* = 0.623). Donor D showed highly statistically significant variability (χ^2_4 = 25.51, *P* < 0.001). Again, the result was driven by a particularly high chromatid aberration frequency in one of the samples received from that donor, removal of which resulted in an intra-individual variation that was not statistically significant (mean = 91.00 ± 8.08, χ^2_3 = 6.09, *P* = 0.193). The results obtained from donors D and F suggested an extra cause for the variation in the yields of chromatid aberrations observed within an individual, out with overdispersion. Intra-individual variability was accounted for by calculating the sum of the values of χ^2 for those individuals sampled on more than one occasion (71.75) and dividing by the total degrees of freedom (38), resulting in a ratio of 71.75:38 (x 1.89). Inter-individual variation was determined, adjusting for overdispersion and intra-individual variability, using χ^2 analysis. A highly statistically significant variation between the donors was observed (χ^2_{26} = 60.31, *P* < 0.001).

Assay reproducibility, blood storage and transportation

Assay reproducibility was investigated by setting up four replicate cultures using blood from a single sample donated by donor N. The results are illustrated in Figure 2.4, together with the results for assay reproducibility following extended blood storage at room temperature for 24 hours (refer to Table 2.3 for sample details). Chi-squared analysis revealed no statistically significant difference in the induced chromatid aberration yields between the four replicate cultures set up on day 0 (χ^2_3 = 0.95, *P* = 0.81) and similarly, no statistically significant difference between the four replicate cultures set up on day 1 (χ^2_3 = 0.68, *P* = 0.88). Comparing induced chromatid aberration yields of all eight cultures also revealed no statistically significant difference (χ^2_7 = 1.96, *P* = 0.96), indicating that storing blood at room temperature for 24 hours prior to culturing has no effect. Table 2.5 provides chromatid aberration frequencies for the eight cultures, mean

chromatid aberration frequencies for day 0 and day 1 and coefficients of variation for the samples.

The effect of storing blood samples at room temperature for 24 hours prior to culturing was investigated further using samples collected from two individuals (donors N and O), over several months. In addition, the effect of temperature on blood storage prior to culturing was analysed (refer to Table 2.3 for sample details). The results are illustrated in Figure 2.5. There was no statistically significant difference in the induced chromatid aberration yield between any of the samples cultured immediately, after 24 hours at room temperature or after 24 hours at 4ºC (χ^2_7 = 19.04, P = 2.37). Chromatid aberration frequencies, mean induced chromatid aberration frequencies, coefficients of variation, χ^2 and P values for all the samples analysed are given in Table 2.6. The effect of transporting blood samples was also investigated using samples received from four donors (donors D, O, K and Q). The results are illustrated in Figure 2.6 (refer to Table 2.3 for sample details). There was no statistically significant effect in transporting blood samples overnight prior to culturing compared to using freshly drawn blood to culture (χ^2_6 = 4.52, P = 4.62). Chromatid aberration frequencies, mean induced chromatid aberration frequencies, coefficients of variation, χ^2 and P values for all the samples analysed are given in Table 2.7.

Table 2.4: Characteristics of study donors, induced chromatid aberration frequencies at different sampling points, mean chromatid aberration frequencies ± S.E. and intra-individuals variations.

			Induced chromatid aberration frequency per 100 cells[1] at different sampling times[2]																Mean ± S.E.[3]	Intra-individual variation	
Donor	Sex	Age	12/03	19/03	03/04	09/04	30/04	14/05	21/05	18/06	02/07	16/07	23/07	17/09	08/10	03/12	14/01	25/05	08/06		χ^2	*P*
A	F	28						87	74	98										86.33 ± 9.09	2.20	0.333
B	F	30						71												71.00 ± 14.28		
C	F	27	93																	93.00 ± 16.34		
D	M	47	76	154	106			105		77										103.60 ± 7.71	25.51	<0.001
F	M	29				137	109	92												112.67 ± 10.39	6.03	0.049
H	F	27	109																	109.00 ± 17.70		
I	M	33																90*		90.00 ± 16.08		
J	F	26				72	68			79										73.00 ± 8.36	0.56	0.756
K	F	34	73	99	99		92	103	77		69									87.43 ± 5.99	8.94	0.177
L	M	48																	93*	93.00 ± 16.34		
M	F	54							110											110.00 ± 17.78		
N	F	36	75	68		97	98	80		83	86	101	63	79	113	82	98			86.38 ± 4.37	18.62	0.098
O	F	48	109		93	107		86	95						92	86				95.43 ± 6.26	3.54	0.738
P	F	21		73				108		78										86.33 ± 9.09	5.46	0.065
Q	F	44			103			87	96											95.33 ± 9.55	0.89	0.641
T	M	20											144							144.00 ± 20.34		
U	M	29																113*		113.00 ± 18.02		
V	M	22											118							118.00 ± 18.41		
W	M	22											119							119.00 ± 18.49		
X	M	54															110			110.00 ± 17.78		
Y	F	33											105							105.00 ± 17.37		
Z	M	48															155			155.00 ± 21.10		
CC	F	23																	77*	77.00 ± 14.87		
DD	M	42																90*		90.00 ± 16.08		
EE	F	21																	114*	114.00 ± 18.10		
FF	F	36																128*		128.00 ± 19.18		
GG	F	21																	59*	59.00 ± 13.02		

[1] Number of gaps and breaks per 100 metaphases. [2] Sample collection (day/month) between March 2001 and January 2002. [3] S.E.: Standard error. * Sample collection (day/month) in June/July 2004.

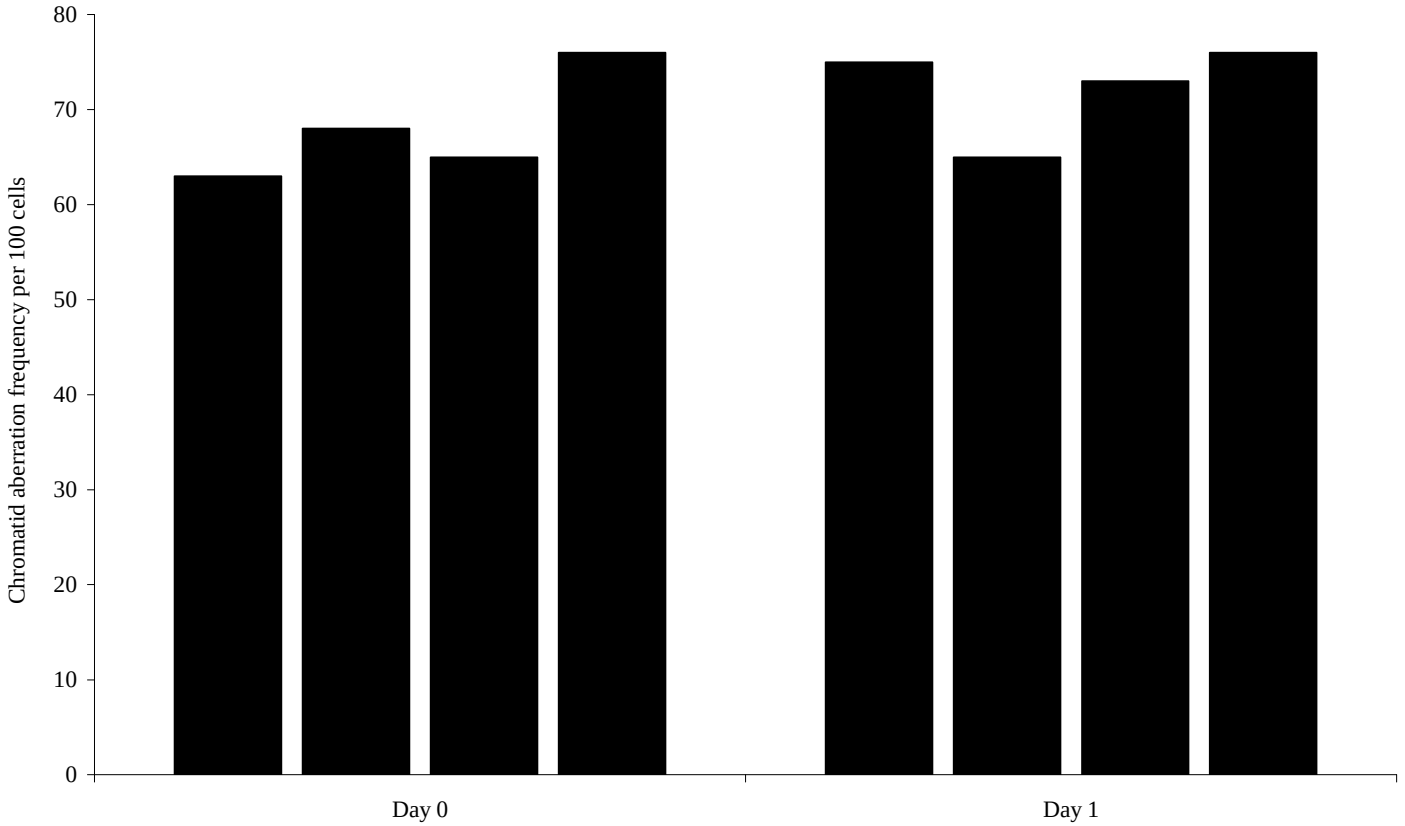


Figure 2.4: Assay reproducibility measured using a single blood sample from donor N. Day 0 and Day 1 represent the chromatid aberration yields (number of gaps and breaks per 100 metaphases) for each of the four replicate cultures set up using fresh blood and blood that had been stored at room temperature for 24 hours, respectively.

Table 2.5: Assay reproducibility using a single blood sample from donor N.

	Chromatid aberration frequency per 100 cells				Mean ± sd[1]	CV[2] (%)	χ^2	*P*
Culture	1	2	3	4				
Day 0	63	68	65	76	68.00 ± 5.72	8.41	0.95	0.81
Day 1	75	65	73	76	72.25 ± 4.99	6.91	0.68	0.88
Comparing all eight cultures					70.13 ± 5.46	7.79	1.96	0.96
Comparing means of day 0 and day 1					70.13 ± 3.01	4.29	0.08	0.77

[1] Standard deviation, [2] Coefficient of variation.

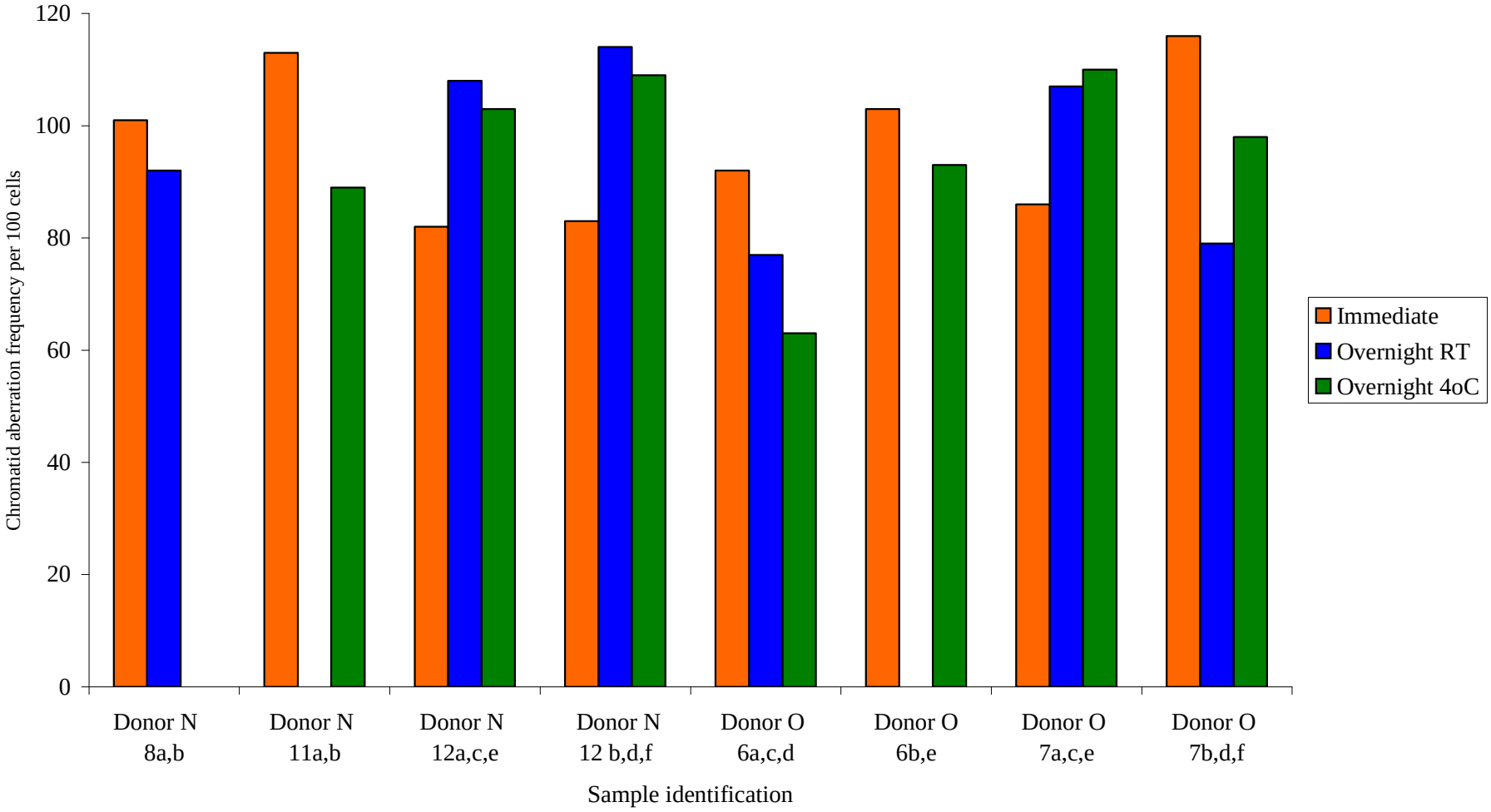


Figure 2.5: The effect of storing blood samples overnight at room temperature and 4°C on chromatid aberration frequencies.

Refer to Table 2.3 for sample identification details (e.g. Donor N, sample 8, fractions a and b).

Table 2.6: The effect of storing blood samples on chromatid aberration frequencies.

Refer to Table 2.3 for sample identification details (e.g. Donor N, sample 8, fractions a and b).

Sample ID	Chromatid aberration frequency per 100 cells			Mean ± sd[1]	CV[2] (%)	χ^2	*P*
	Immediate	RT	4°C				
Donor N – sample 8a,b	101	92	No sample	96.50 ± 6.36	6.59	0.28	0.60
Donor N – sample 11a,b	113	No sample	89	101.00 ± 16.97	16.80	1.88	0.17
Donor N – sample 12a,c,e	82	108	103	97.67 ± 13.80	14.13	2.56	0.28
Donor N – sample 12b,d,f	83	114	109	102.00 ± 16.64	16.32	3.57	0.17
Donor O – sample 6a,c,d	92	77	63	77.33 ± 14.50	18.75	3.58	0.17
Donor O – sample 6b,e	103	No sample	93	98.00 ± 7.07	7.22	0.34	0.56
Donor O – sample 7a,c,e	86	107	110	101.00 ± 13.08	12.95	2.23	0.33
Donor O – sample 7b,d,f	116	79	98	97.67 ± 18.50	18.94	4.61	0.10

[1] Standard deviation, [2] Coefficient of variation.

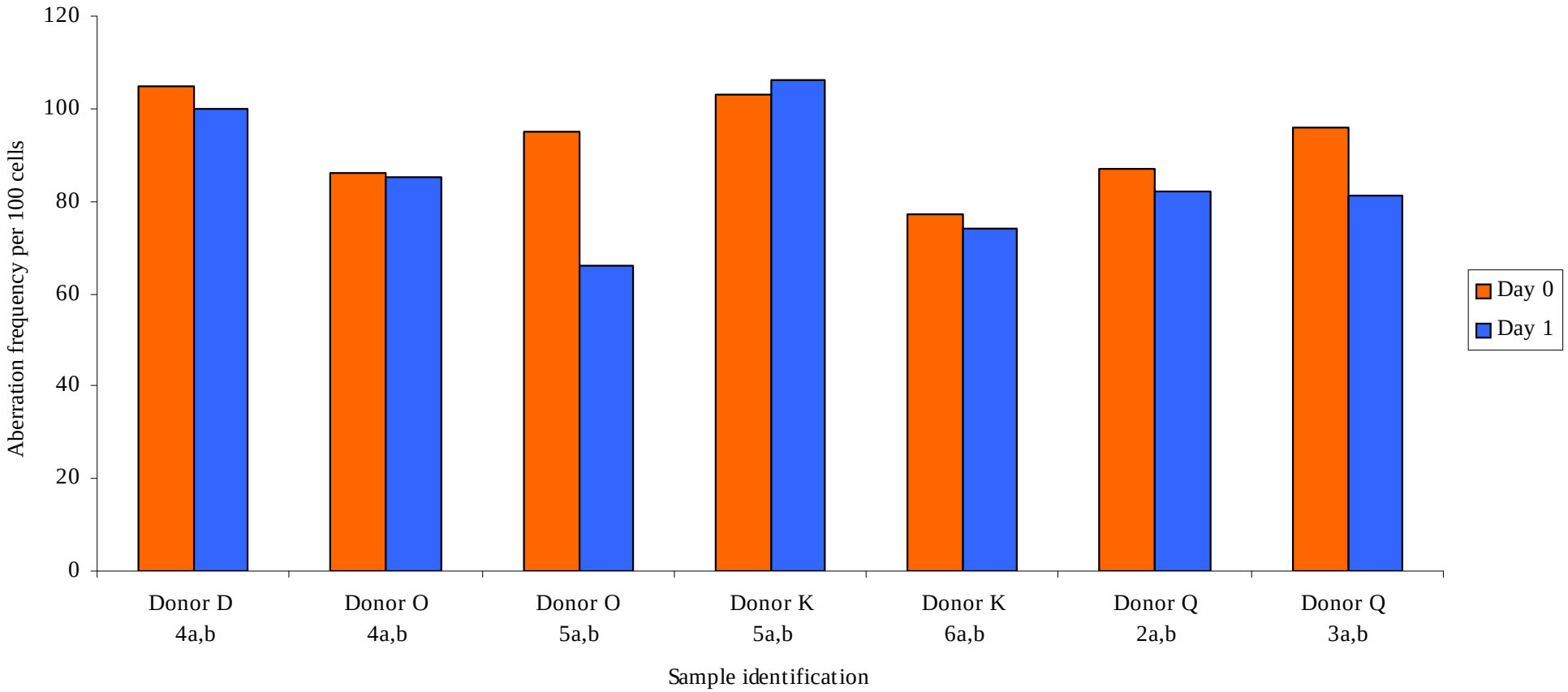


Figure 2.6: The effect of transporting blood samples on chromatid aberration frequencies. Day 0 represents blood cultures set up immediately. Day 1 represents those that were transported. Refer to Table 2.3 for sample identification details (e.g. Donor D, sample 4, fractions a and b).

Table 2.7: The effect of transporting blood samples on G_2 induced chromosome chromatid aberration frequencies.

Refer to Table 2.3 for sample identification details (e.g. Donor D, sample 4, fractions a and b).

Sample ID	Chromatid aberration frequency per 100 cells		Mean ± sd[1]	CV[2] (%)	X^2	*P*
	Immediate culture	Transportation				
Donor D – sample 4a,b	105	100	102.50 ± 3.54	3.45	0.08	0.78
Donor O – sample 4a,b	86	85	85.50 ± 0.71	0.83	0.00	0.95
Donor O – sample 5a,b	95	66	80.50 ± 20.51	25.47	3.44	0.06
Donor K – sample 5a,b	103	106	104.50 ± 2.12	2.03	0.03	0.87
Donor K – sample 6a,b	77	74	75.50 ± 2.12	2.81	0.04	0.84
Donor Q – sample 2a,b	87	82	84.50 ± 3.54	4.18	0.10	0.76
Donor Q – sample 3a,b	96	81	88.50 ± 10.61	11.98	0.84	0.36

[1] Standard deviation, [2] Coefficient of variation.

Examining radiosensitivity

Distributions of chromatid aberration frequencies amongst the WRI validation study population are illustrated in Figure 2.7. The 90^{th} percentile cut-off point to discriminate between a radiosensitive and non-radiosensitive response was determined (using Microsoft Excel) to be 122.6 chromatid aberrations per 100 cells, resulting in 11.11% of the study population displaying a radiosensitive response (3 individuals).

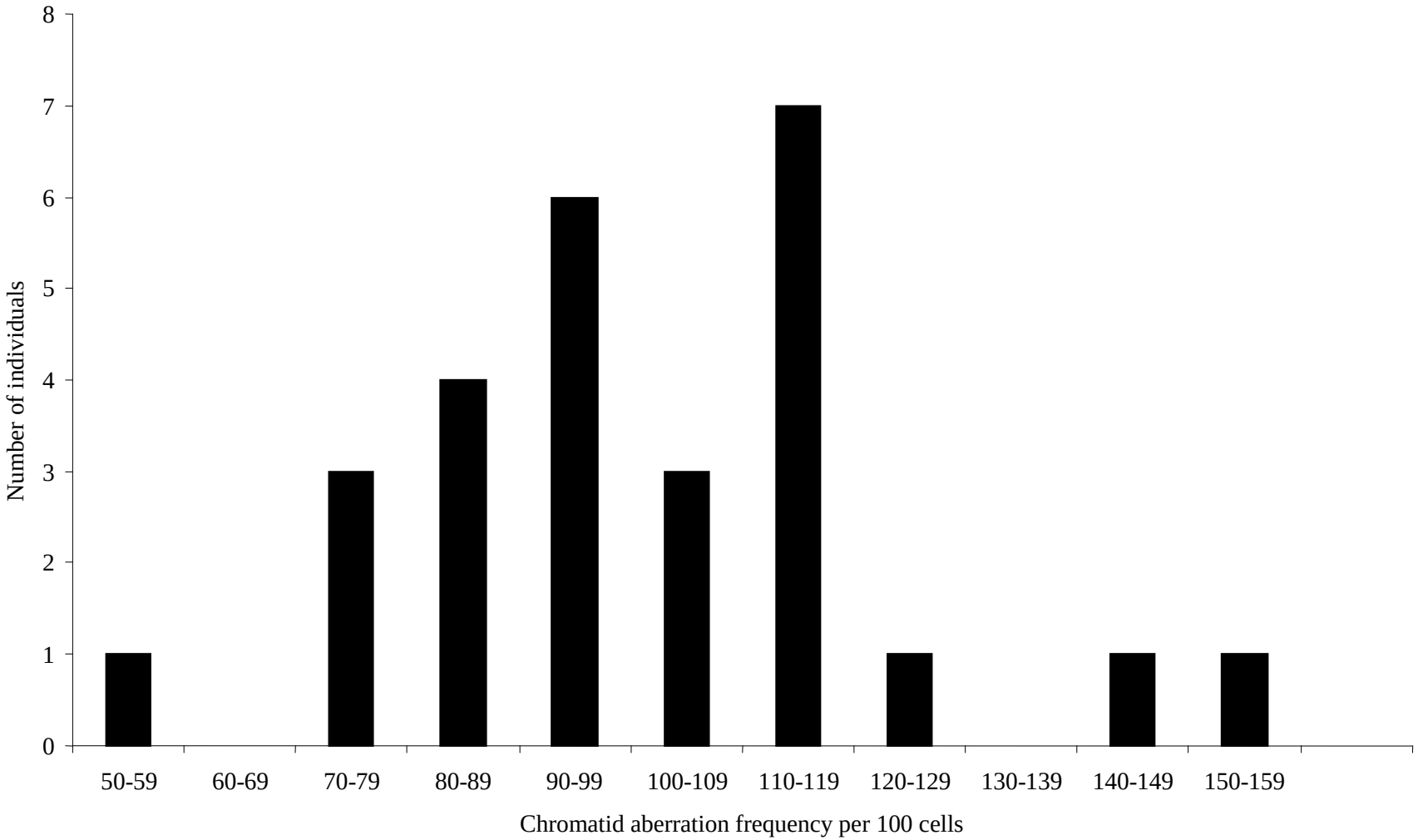


Figure 2.7: Distribution of G_2 induced chromatid aberration frequencies for the WRI validation study population.

Discussion

Assay reproducibility, blood storage and transportation

To date, a number of studies have reported on G_2 radiosensitivity (radiation induced chromatid aberration levels) in normal populations and, although it may not be feasible to directly compare results from various laboratories due to systematic differences between studies e.g. sampling effects or different scoring criteria, as long as standard procedures are adopted throughout, a valid qualitative comparison can be made. In the present validation study, the range of induced chromatid aberration frequencies observed was found to be similar to those of other studies implementing comparable laboratory techniques and scoring criteria (Baria *et al.*, 2002; Howe *et al.*, 2005; Riches *et al.*, 2001). In addition, analysis of multiple cultures set up in tandem from a single blood sample provided good evidence of a high standard of intrinsic assay reproducibility in the Westlakes laboratory, similar to the findings of Vral *et al.* (2002). Whilst assay reproducibility had been confirmed, previous studies reported that the G_2 assay requires stringent technical conditions to produce reproducible results in terms of variability in induced chromatid aberration yields, with problems possibly associated with blood sample storage and transport conditions (Bryant *et al.*, 2002; Scott *et al.*, 1999; Scott *et al.*, 1996). Indeed, experiments at the University of St Andrews indicated that samples from some individuals exhibited a reduced G_2 score if blood was stored overnight (Bryant *et al.*, 2002). However, in another study, no difference was found in results for blood samples taken from normal individuals and set up at various times up to 24 hours (Scott *et al.*, 1999). In addition, no significant differences were found in the level of chromatid aberrations produced from blood samples stored between 2 and 24 hours after bleeding and kept at temperatures varying from 20-37°C (Scott *et al.*, 1996). Nevertheless, in this

validation study, no statistically significant differences were found between blood samples cultured immediately and those with extended storage overnight at either normal ambient laboratory temperature or at 4°C. Transport of blood samples over long distances has been reported to influence G_2 score (Scott *et al.*, 1999), whilst transportation over short distances has been suggested to have no effect on reproducibility (Riches *et al.*, 2001; Roberts *et al.*, 1999). In this study, all blood samples were drawn at the West Cumberland Hospital and were transported a distance of approximately 2km to Westlakes Research Institute prior to culturing. No transportation effect was observed in these samples or in samples purposely travelled further distances over longer periods of time to test for possible effects.

Intra- and inter-individual variation

On examination of intra-individual variation, seven out of nine donors displayed a non-statistically significant result when using χ^2 analysis, indicating that G_2 chromosomal radiosensitivity was relatively stable and reproducible. Of the two donors displaying statistically significant results, removal of a single sample point with a high G_2 chromatid aberration score from each of the individuals resulted in non-significance for both. On further examination of these two 'high' G_2 values, comparison with samples taken from other individuals on the same day failed to reveal other unusual scores, indicating that experimental error was not the likely cause but rather intrinsic factors in the individuals themselves. Indeed, significant intra-individual variability has been reported by others validating the G_2 assay (Howe *et al.*, 2005; Vral *et al.*, 2002; Vral *et al.*, 2004). For the study by Howe *et al.*, three out of four donors produced coefficients of variation between 4.58 and 5.09%, being consistent with values reported by Scott *et al.* (1999). However, a fourth donor showed a variation of 22.9% and was statistically different to the

abovementioned three. From this, it was concluded that the assessment of individual radiosensitivity should not be based on a single blood sample but rather multiple samples may be necessary to obtain reliable conclusions. Similarly, Vral *et al*. (2002) examined 14 individuals over a period of one year, with two individuals providing nine samples each. Intra-individual variation determined from these two donors was found to be similar to the inter-individual variability observed, indicating that the assay was unlikely to detect any real differences in chromosomal radiosensitivity that exist between most normal individuals within a population. In the 2004 study, an extension of the 2002 study involving analysis of 14 donors over a period of three years, Vral *et al*. showed intra-individual variation that was not statistically significant in three out of four repeatedly sampled individuals (between 4 and 15 repeats). However, comparison of intra- and inter-individual variation again failed to reveal a significant difference, confirming the findings of Baria *et al.* (2002) who recorded an intra-individual variation of 18.6% and an inter-individual variation of 19.2%. In contrast, previous studies by the same group reported intra-individual variation in the range of 7-10% and inter-individual variation in the range of 15-20% (Baria *et al.*, 2001; Papworth *et al.*, 2001; Roberts *et al.*, 1999). Since good assay reproducibility had been determined in the present study and it was possible to exclude experimental error as the cause of the two high G_2 scores affecting intra-individual variation, other possible causal explanations were sought. Factors such as changes in diet and immune status have previously been reported to affect blood cell and serum composition, which in turn may influence the response of a sample to ionising radiation (Fenech, 1999). In addition, changes in hormone levels have been shown to have a profound effect upon radiosensitivity both *in vivo* and *in vitro* (Roberts *et al.*, 1997), although this has been disputed in more recent studies (Baeyens *et al.*, 2005). Further studies, investigating the effects of such factors on *in vitro* chromosomal radiosensitivity

would be useful. In terms of inter-individual variation, a highly statistically significant effect was observed in this validation study, leading to the conclusion that the G_2 assay would be a useful biomarker of chromosomal radiosensitivity in population studies but due to the variation observed in a small number of donors when repeatedly sampling, it should not be used to determine individual sensitivity. The results of this validation study have been published in Smart *et al.* (2003).

CHAPTER 3

Examining G_2 chromosomal radiosensitivity in childhood and adolescent cancer survivors, their partners and offspring

Introduction

The relationship between radiosensitivity and cancer predisposition and whether the cellular response can be inherited was investigated using the G_2 assay of chromosomal radiosensitivity on the survivors of childhood and adolescent cancer and their offspring whilst using the partners of the cancer survivors as a control group.

Materials and Methods

The Danish trio study population

Blood samples were obtained for this pilot study from the families of 28 Danish childhood and adolescent cancer survivors who had been treated with radiotherapy. Each set of family samples included peripheral blood from the survivor of childhood and adolescent cancer, their partner and their offspring and while each family may have consisted of more than one offspring, a family unit was referred to as a "trio". Details of the donors are provided in Table 3.1.

Nordic countries are unique in that they operate population-based health registries that can be accessed with individual identification numbers assigned to each citizen at birth. All patients diagnosed with cancer before the age of 20 years between the beginning of 1943

through to the end of 1996, and who survived until a fertile age of 15 were identified by Dr Jeanette Falck Winther of the Danish Cancer Society, using files from the nation-wide population based Danish Cancer Registry. Patients further eligible for study were those who were alive on, or born after, 1st April 1968 when the national Central Population Register (CPR) was established and all citizens were assigned their personal identification number (PIN). This number incorporates information such as sex and date of birth and also permits accurate linkage of information between registries. The CPR provides information on the date of childhood cancer diagnosis, the type of cancer suffered and information about any secondary primary cancers, radiotherapy/chemotherapy received, and by linkage, can be used to identify partners and offspring of cancer survivors. This produced a cohort of 4,676 childhood and adolescent cancer survivors (2655 males and 2021 females). The 28 families suitable for entrance into the pilot study were selected from the remaining cohort by Dr Falck Winther, who also co-ordinated collection of the blood samples at the 'Late Effect Clinic' in University Hospital, Copenhagen. Final inclusion criteria included the childhood and adolescent cancer survivors having received moderate to high doses of scattered radiation to the gonads (prerequisite for a parallel study investigating the effects of radiation to the gonads on minisatellite mutation frequency), having live offspring and having been treated at either the Rigshospitalet (State Hospital) in Copenhagen and hence already familiar with the Late Effect Clinic for childhood and adolescent cancer survivors held there, or the Aarhus Kommunehospital (Community Hospital) in Jutland.

Danish trio sample collection

Eligible survivors were contacted by means of a letter to determine their willingness to participate in the study. The Danish Scientific Ethical Committee and the Danish Data

Protection Agency reviewed and approved the blood study protocol. Families were invited to the Late Effect Clinic to have bloods drawn and on arrival were further informed about the study by a medical doctor. Each family signed an informed consent form (refer to Appendix C) and blood samples received were coded to ensure anonymity. In addition, information on dosimetry, cancer type, medical treatment and lifestyle information was obtained through a short questionnaire (refer to Appendix C). A total of 23.5ml of blood was drawn from each individual, with 10ml being drawn into two lithium heparin vacutainers (BD Vacutainer Systems, Ref. 367684) for transportation to WRI. The remainder of the blood sample was processed and stored at the Institute of Cancer Biology, Denmark, for possible future studies where viable lymphocytes were frozen and stored together with plasma samples, and DNA extracted. Figure 3.1 illustrates a flow chart for blood samples. Before each family left the clinic, they received a small information pamphlet (refer to Appendix C) detailing the study, reimbursement of their travel expenses and a small present for each child. A total of 100 samples were collected and these were transported to the UK in 10 shipments over a period of 13 months. Blood samples were drawn from participating families on Monday and transported overnight directly from Copenhagen to WRI by DHL courier, arriving Tuesday morning. Vacutainers were stored in screw-cap polyethylene tubing throughout transportation, packed with thick, absorbent cotton wool to avoid breakage and absorb spillage. Tubes were placed into a closed plastic coated envelope to allow easy inspection at customs, and, to ensure samples had not been exposed to X-rays, a piece of dental film was included with each shipment. To avoid extreme temperature fluctuations, samples were not taken into the hull of the plane but instead kept with the courier at all times. It took approximately 16 hours from the time the samples were drawn to arrive at WRI, upon which cell culture was set up immediately and the G_2 assay performed as described in Chapter 2 (*Determining G_2 radiosensitivity; The G_2*

assay). It was possible to collect only single blood samples from the Danish population as they could not be re-sampled due to the ethical constraints of the study. Thus, triplicate blood cultures were set up for the G_2 assay with two cultures irradiated, the second culture acting as a back up in case of experimental error and one culture acting as the non-irradiated control. Metaphase slides were prepared and scored as also described in Chapter 2 (*Determining G_2 radiosensitivity; Slide preparation*).

Measuring mitotic inhibition

To determine if the efficacy of the G_2 checkpoint control was reduced in the childhood and adolescent cancer survivors or their offspring compared with healthy controls and to determine whether this might be a contributory factor in enhanced chromosomal radiosensitivity, the degree of mitotic inhibition was assessed in all populations after exposure of lymphocytes to X-rays. Mitotic indexes for each sample were determined by calculating the percentage of mitotic cells per 1000 unselected mononuclear cells using the same irradiated and non-irradiated slides scored for chromatid-type aberrations in the G_2 assay. Mitotic inhibition was then calculated as the percentage reduction in mitotic index in irradiated compared with non-irradiated samples.

Transportation and internal assay controls

Each shipment of samples received from Denmark also contained blood from one healthy Danish volunteer to act as a control for the transportation system. This volunteer remained consistent throughout the study. In addition, repeat samples from one consistent, healthy adult WRI volunteer were collected and assayed in parallel to the samples received from Denmark, to act as an internal experimental control. These samples were collected at West Cumberland Hospital on a Monday morning concurrent with the samples collected in

Denmark and transported back to WRI, where they were stored at room temperature overnight before being cultured for the G_2 assay. For the internal experimental control, two sample points were missed (27/05/02 and 03/06/02) due to other commitments held by this member of staff. Details of the donors and samples received are given in Table 3.2.

Table 3.1: Characteristics of trio donors.

Family ID (shipment date)	Study ID	Family member		Age at sampling (years)	Age at diagnosis (years)	Diagnosis of survivor
1	0101	Father	Partner	28		
(11/03/02)	0102	Mother	Survivor	35	15	Hodgkin's disease
	0103	Child	Male	2		
	0104	Child	Female	7 months		
2	0201	Father	Partner	31		
(18/03/02)	0202	Mother	Survivor	34	11	Hodgkin's disease
	0203	Child	Male	3		
	0204	Child	Male	12 months		
3	0301	Father	Survivor	34	9	Rhabdomyosarcoma
(06/05/02)	0302	Mother	Partner	32		
	0303	Child	Male	8		
	0304	Child	Female	5		
4	0401	Father	Partner	39		
(06/05/02)	0402	Mother	Survivor	36	15	1. Primary: Hodgkin's disease
					30	2. Primary: Thyroid cancer
	0403	Child	Female	3		
5	0501	Father	Survivor	36	10	Hodgkin's disease
(27/05/02)	0502	Mother	Partner	31		
	0503	Child	Male	9		
	0504	Child	Male	7		
	0505	Child	Female	17 months		No sample provided
6	0601	Father	Partner	36		
(27/05/02)	0602	Mother	Survivor	33	0.1	Teratoma
	0603	Child	Female	14		
	0604	Child	Male	9		
	0605	Child	Female	6		
7	0701	Father	Partner	30		
(27/05/02)	0702	Mother	Survivor	29	19	Hodgkin's disease
	0703	Child	Female	2		
8	0801	Father	Survivor	32	0.6	Neuroblastoma
(03/06/02)	0802	Mother	Partner	33		
	0803	Child	Female	2		
9	0901	Father	Survivor	33	7	Wilms' tumour
(03/06/02)	0902	Mother	Partner	36		
	0903	Child	Male	4		
	0904	Child	Male	3		
	0905	Child	Male	13 months		
10	1001	Father	Partner	26		
(09/12/02)	1002	Mother	Survivor	25	4	Wilms' tumour
	1003	Child	Male	3		
11	1101	Father	Survivor	30	8	Non-Hodgkin's lymphoma
(09/12/02)	1102	Mother	Partner	29		
12	1201	Mother	Survivor	25	14	Hodgkin's disease
(09/12/02)	1202	Father	Partner	31		
	1203	Child	Male	11 months		
13	1301	Mother	Survivor	30	20	Lymphoepithelioma
(16/12/02)	1302	Father	Partner	36		
	1303	Child	Male	4 months		
14	1401	Father	Survivor	43	17	Ewing's sarcoma
						Final diagnosis: Osteomyelitis
(16/12/02)	1402	Mother	Partner	37		
	1403	Child	Female	9		

	1404	Child	Female	6		
15	1501	Father	Survivor	35	19	Pineocytoma
(16/12/02)	1502	Mother	Partner	33		
	1503	Child	Female	7		
16	1601	Father	Partner	35		
(24/03/03)	1602	Mother	Survivor	33	20	Hodgkin's disease
	1603	Child	Male	5		
17	1701	Father	Survivor	24	17	Germinoma
(24/03/03)	1702	Mother	Partner	23		
	1703	Child	Male	19 months		
18	1801	Father	Survivor	35	19	Malignant schwannoma
(24/03/03)	1802	Mother	Partner	31		
	1803	Child	Female	3		
	1804	Child	Male	2		
19	1901	Mother	Partner	35		
(31/03/03)	1902	Father	Survivor	35	17	Hodgkin's disease
	1903	Child	Female	12		
	1904	Child	Female	10		
20	2001	Father	Partner	33		
(31/03/03)	2002	Mother	Survivor	33	17	Hodgkin's disease
	2003	Child	Female	3		
	2004	Child	Male	3		
21	2101	Mother	Survivor	35	19	Hodgkin's disease
(31/03/03)	2102	Father	Partner	37		
	2103	Child	Male	9		
	2104	Child	Male	5		No sample provided
	2105	Child	Male	3		No sample provided
22	2201	Mother	Partner	36		
(31/03/03)	2202	Father	Survivor	31	1	Wilms' tumour
	2203	Child	Female	2		
23	2301	Father	Survivor	32	5	Wilms' tumour
(07/04/03)	2302	Mother	Partner	36		
	2303	Child	Male	6		
	2304	Child	Male	13 months		
24	2401	Father	Partner	37		
(07/04/03)	2402	Mother	Survivor	36	14	1. Primary: Malignant lymphoma
					33	2. Primary: Breast cancer
	2403	Child	Female	13		
	2404	Child	Male	9		
25	2501	Father	Partner	37		
(07/04/03)	2502	Mother	Survivor	36	1	Neuroblastoma
	2503	Child	Female	9		
26	2601	Father	Partner	43		
(07/04/03)	2602	Mother	Survivor	37	19	Hodgkin's disease
	2603	Child	Female	13		
	2604	Child	Male	10		
27	2701	Mother	Survivor	36	2	Wilms' tumour
(07/04/03)	2702	Father	Partner	40		
	2703	Child	Male	7		
	2704	Child	Female	5		
28	2801	Father	Partner	35		
(07/04/03)	2802	Mother	Survivor	34	2	Wilms' tumour
	2803	Child	Male	3		
	2804	Child	Male	3		

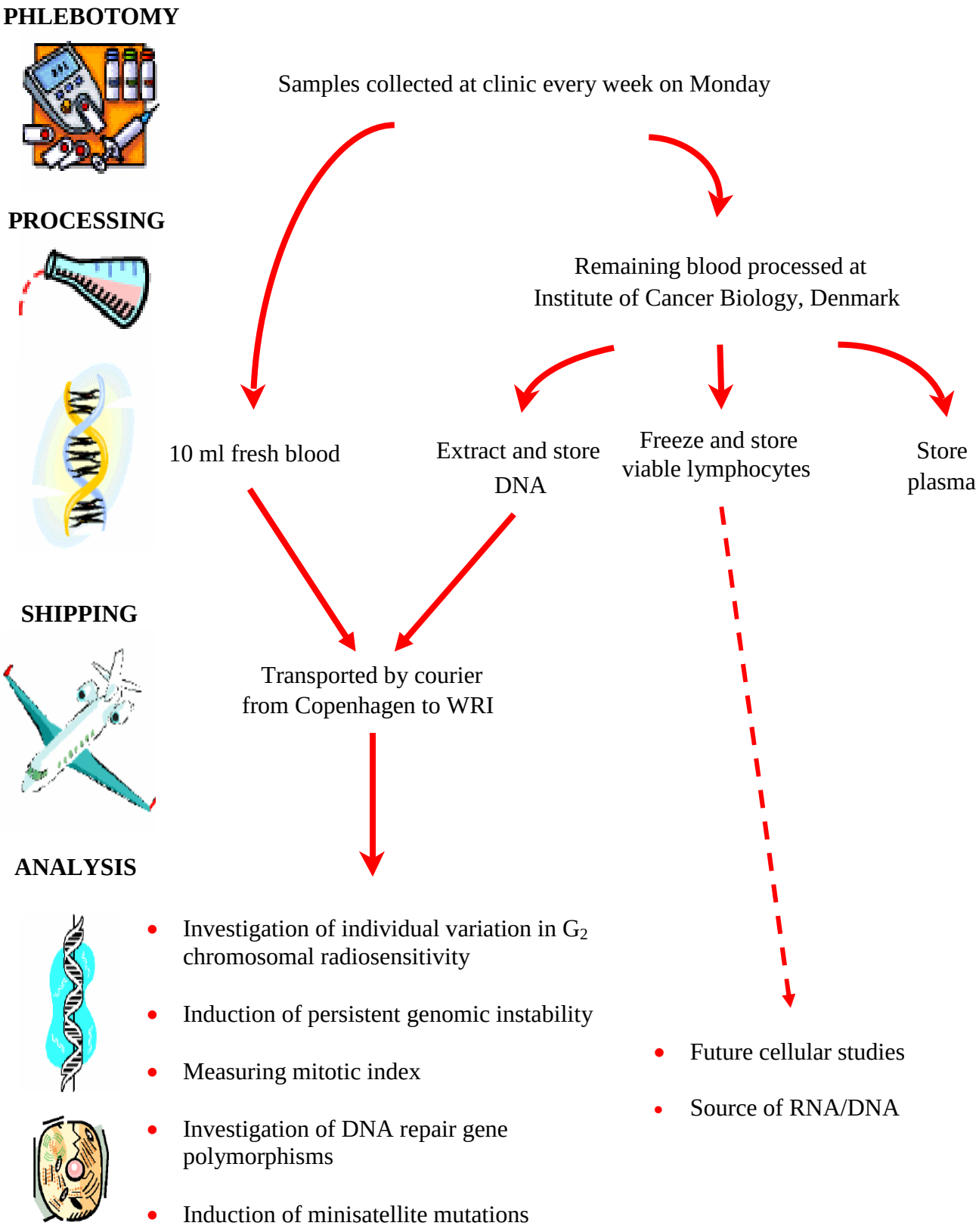


Figure 3.1: Flow chart for Danish trio blood samples.

Table 3.2: Details of transportation and WRI internal assay control donors and samples provided.

Donor	Sex	Age at sampling (years)	Sample shipment dates from Denmark									
			11/03/02	18/03/02	06/05/02	27/05/02	03/06/02	09/12/02	16/12/02	24/03/03	31/03/03	07/04/03
Transport Control	F	37	4	4	4	4	4	4	4	4	4	4
Internal Control	F	37	4	4	4	7	7	4	4	4	4	4

Statistical analysis

Analysis of aberration distribution

As described in Chapter 2 (*Statistical analysis; Analysis of aberration distribution*), Poisson statistics were used to study the distribution of chromatid aberrations amongst metaphase cells for the different populations (childhood and adolescent cancer survivors, partners, offspring, WRI internal assay and transport control populations). Ratios of variance to mean for each sample provided by a donor were calculated and population ratios determined using the average value.

Estimating intra- and inter-individual variability

Chi-squared analysis was used to investigate homogeneity of repeat sampling in the transport control and WRI internal control populations and overdispersion of distributions of chromatid aberrations amongst metaphase cells corrected for in each population as described in Chapter 2 (*Statistical analysis; Estimating intra- and inter-individual variability*). Inter-individual variation was also estimated using χ^2 analysis with overdispersion taken into account as above.

Mean and median induced aberration yields

Mean chromatid aberration frequencies were calculated for each population by dividing the total number of aberrations observed by the total number of cells scored and normalising to 100 cells scored. Median chromatid aberration yields were determined using the Minitab15 statistical software package and the non-parametric Mann-Whitney U-test used to compare values between populations.

Standard errors

In all cases, standard errors were calculated by adjusting for overdispersion of chromatid aberrations using the appropriate compensation factor equated for each population as described in Chapter 2 (Z = average value of ratio of variance to mean, calculated for each population). In addition, for the WRI internal assay and transport control populations (where repeat sampling had occurred), any additional intra-individual variation introduced was also compensated for as described in Chapter 2 (*Statistical analysis; Standard errors*). Standard errors were thus calculated according to the formula √(Number of aberrations x Z x Y), and normalised to 100 cells scored.

Determining radiosensitivity

The proportion of radiosensitive individuals within each population was determined by using a cut –off value of the 90^{th} percentile of induced aberration frequencies per 100 cells, as suggested by Scott (Roberts *et al.*, 1999; Scott *et al.*, 1999), for the partner control population. Fisher's exact tests were used to compare the proportions of radiosensitive individuals between the populations using the web-page http://www.matforsk.no/ola/fisher.htm.

Determining heritability of radiosensitivity

Heritability of G_2 chromosomal radiosensitivity amongst the donor groups was assessed by two methods. Initially, Fisher's exact tests populations (using the web-page http://www.matforsk.no/ola/fisher.htm) were used to compare the proportions of radiosensitive and non-radiosensitive offspring split into groups according to the response of the parents. Secondly, segregation analysis was performed using the Statistical Analysis for Genetic Epidemiology software (SAGE, 2004) and heritability was assessed

using standard quantitative genetic variance component analysis implemented in the SOLAR software package and was obtained as the ratio of the additive genetic variance to the total phenotypic variance. This was performed by Dr. Ranajit Chakraborty, Centre for Genome Information, University of Cincinnati College of Medicine, Cincinnati, Ohio.

Assessing mitotic inhibition

Analysis of variance (one way ANOVA) was used to compare mean mitotic indices between the three groups and mean mitotic inhibition. Comparison of median mitotic indices between the groups and median mitotic inhibition was performed using the non-parametric Mann-Whitney U test. Spearmans rank correlation analysis was used to examine the relationship between G_2 chromosomal radiosensitivity and mitotic indices for each group and also G_2 chromosomal radiosensitivity and mitotic inhibition. All analyses were performed using the Mintab15 statistical software package.

Results

Internal assay and transport control data

Chromatid aberration frequencies for the transport and internal assay controls are given in Table 3.3. Some of the samples received in shipment eight failed to culture successfully and thus the associated transport and internal assay controls plus all families received in this shipment, were excluded from the study. This gave a total of six samples from the internal assay control and nine samples from the transport control available for analyses.

As with the validation study population, analysis of chromatid aberration distributions amongst the cells analysed for the internal assay control again revealed an overdispersion as compared to a Poisson distribution, with an average ratio of variance to mean of 1.67. Thus, overdispersion was taken into account by adjusting the expected values for the yields of chromatid aberrations per 100 metaphases analysed in subsequent calculations, by this factor. The internal assay control produced a mean chromatid aberration frequency of 108.00 ± 2.44 per 100 cells and exhibited no statistically significant difference between the samples collected (χ^2_5 = 5.95, P = 0.311), indicating intra-individual variation was minimal and there was no intrinsic assay effect. In determining the standard error for the mean induced chromatid aberration frequency, any intra-individual variation that was observed was compensated for by calculating the sum of the values of χ^2 (5.95) and dividing by the total degrees of freedom (5), resulting in a ratio of 5.95:5 (x 1.19).

In contrast to the internal assay control, the samples provided by the transport control donor did show a statistically significant difference (χ^2_8 = 25.08, P = 0.002). Again, analysis of chromatid aberration distributions amongst the cells analysed revealed an

overdispersion compared to Poisson distribution, with an average ratio of variance to mean of 1.73 and this was taken into consideration by adjusting the expected values for the yields of chromatid aberrations analysed in subsequent calculations appropriately. The female volunteer who provided the transport samples became pregnant during the study and was pregnant from shipment six through to ten. Re-examination of the samples pre-pregnancy and during pregnancy revealed differences in the intra-individual variations observed. Pre-pregnancy sample analysis (shipments 1–5) revealed no statistically significant difference between the samples ($\chi^2_4 = 6.92$, $P = 0.140$) and a mean induced chromatid aberration frequency of 82.00 ± 3.24 per 100 cells, whilst analysis of the samples provided during pregnancy (shipments 6–10) showed a statistically significant difference ($\chi^2_3 = 14.77$, $P = 0.002$) and a mean induced chromatid aberration frequency of 97.50 ± 6.87 per 100 cells (refer to Table 3.4), suggesting a pregnancy effect on the yield of G_2 chromatid aberrations observed.

Table 3.3: G_2 induced chromatid aberration frequencies for the transportation and WRI internal assay control donors.

Donor	Induced aberration frequency per 100 cells for each sample shipment received from Denmark[1]										Mean ± S.E.[2]
	11/03/02	18/03/02	06/05/02	27/05/02	03/06/02	09/12/02	16/12/02	24/03/03	31/03/03	07/04/03	
Transport Control	91	59	89	99	72	98	122	-	111	59	88.89 ± 2.44
Internal Control	91	91	-	-	-	110	117	-	111	128	108.00 ± 2.44

- data not available, [1] Sample shipments 1–10 collected between 11/03/02 and 07/04/03, [2] S.E.: Standard error.

Table 3.4: Analysis of transportation control samples pre-pregnancy and during pregnancy.

Sample ID	Average ratio of variance to mean	χ^2	*P*	Intra-individual variation factor	Mean aberration frequency ± S.E.[1]
All samples	1.73	25.08	0.002	3.14	88.89 ± 2.44
Pre-pregnancy samples	1.85	6.92	0.140	1.73	82.00 ± 3.24
During pregnancy samples	1.57	14.77	0.002	4.92	97.50 ± 6.87

[1] S.E.: Standard error.

Childhood and adolescent cancer survivor, partner and offspring data

The G_2 assay was performed on survivors of childhood and adolescent cancer, their partners and their offspring (so-called 'trios') as detailed above. The resulting chromatid aberration frequencies are given in Table 3.5. As previously mentioned, some of the samples received in shipment eight failed to culture successfully and this resulted in the exclusion of all of the families received in this shipment (families 16, 17, 18) from the study. A further family was excluded as no offspring samples were available for analyses (family 11) and another family was excluded because the final diagnosis of the cancer survivor was not actually cancer (family 14). Therefore, G_2 chromatid aberration frequencies were available for 23 out of a potential of 28 families, giving a total of 84 samples.

Initial analyses concentrated on examining the distributions of chromatid aberrations amongst the three groups of donors. Overdispersion compared to a Poisson distribution was observed in all three groups with average ratios of variance to mean of 1.71, 1.76 and 1.54 for the cancer survivors, partners and offspring respectively. Mean and median G_2 induced chromatid aberration frequencies were determined for each group of donors (refer to Table 3.6), with overdispersion taken into account by the appropriate variance factor when calculating the standard errors. Comparison of the mean induced chromatid aberration frequencies highlighted similarities between the cancer survivor and partner control group but that achieved for the offspring group was higher. This was mainly driven by one extremely elevated induced yield of 406 chromatid aberrations per 100 cells (donor 605) (refer to Table 3.5). This result was verified by scoring the duplicate irradiated blood culture set up from this sample and also by checking the scores of other samples irradiated at the same time in order to rule out any error in the irradiation

procedure. Removal of this sample from the analysis resulted in a lower mean chromatid aberration frequency, more comparable to those of the cancer survivor and the partner control groups. Comparison of the median induced chromatid aberration frequencies revealed no statistically significant difference to that of the partner control group. In addition, comparison of the median induced chromatid aberration frequencies between the WRI validation study group and the partner control group also revealed no statistically significant difference ($P = 0.058$). However, significant differences were observed when comparing that of both the cancer survivor group ($P = 0.009$) and the offspring group ($P = 0.001$) to the WRI validation study group (refer to Table 3.6).

Determining G_2 chromosomal radiosensitivity

Using the 90th percentile of the chromatid aberration frequencies for the partner control group in order to determine between a radiosensitive/non-radiosensitive response, produced a cut-off point of 162.4 chromatid aberrations per 100 cells. This resulted in approximately 0% (0/23), 13% (3/23) and 18% (7/38) of cancer survivors, partner controls and offspring displaying enhanced radiosensitivity, respectively. There was no statistical significance in the proportion of radiosensitive individuals between the partner control group and either the cancer survivors ($P = 0.233$) or the offspring group ($P = 0.729$), indicating that cancer survivors neither display elevated radiosensitivity, nor was the radiosensitivity phenotype inherited (refer to Table 3.7). However, when using the 90th percentile of the WRI validation study group (122.6 chromatid aberrations per 100 cells) as the radiosensitivity cut-off point, examination of the sensitivity profiles revealed statistically significant differences between the proportion of sensitive individuals in the WRI group and the cancer survivor group ($P = 0.002$) and also in the WRI group and the offspring group ($P < 0.001$), whilst no statistical significance was found between the

proportion of sensitive individuals in the WRI group and the partner control group (P = 0.084) (refer to Table 3.7). Distributions of the chromatid aberration frequencies for all donor groups are illustrated in Figure 3.2.

Table 3.5: Chromatid aberration frequencies for survivors of childhood and adolescent cancer, their partners and offspring.

Survivor						Partner			Offspring [1]		
Family ID	Gender	*Diagnosis of primary cancer*	Age at diagnosis (years)	Age at time of sampling (years)	Aberration frequency per 100 cells	Gender	Age at time of sampling (years)	Aberration frequency per 100 cells	Gender	Age at time of sampling (years)	Aberration frequency per 100 cells
1	F	Hodgkin's disease	15	35	123	M	28	84	M	2	89
									F	7months	146
2	F	Hodgkin's disease	11	34	126	M	31	86	M	3	136
									M	12months	80
3	M	Rhabdomyosarcoma	9	34	105	F	32	115	M	8	92
									F	5	108
4	F	Hodgkin's disease	15	36	101	M	39	164	F	3	87
		Thyroid cancer [2]	30								
5	M	Hodgkin’s disease	10	36	74	F	31	155	M	9	124
									M	7	139

									F	17months	No sample
6	F	Teratoma	0.1	33	161	M	36	114	F	14	164
									M	9	157
									F	6	406
7	F	Hodgkin's disease	19	29	109	M	30	101	F	2	105
8	M	Neuroblastoma	0.6	32	134	F	33	108	F	2	164
9	M	Wilms' tumour	7	33	132	F	36	114	M	4	88
									M	3	98
									M	13months	114
10	F	Wilms' tumour	4	25	81	M	26	88	M	3	115
11	M	Non- Hodgkin's lymphoma	8	30	115	F	29	95			No sample
12	F	Hodgkin's disease	14	25	130	M	31	115	M	11months	76
13	F	Lymphoepithelioma	20	30	126	M	36	101	M	4mths	93
14	M	Osteomyelitis	17	43	82	F	37	122	F	9	139
									F	6	118

15	M	Pineocytoma	19	35	98	F	33	156	F	7	129
16	F	Hodgkin's disease	20	33	Failed	M	35	Failed	M	5	Failed
17	M	Germinoma	17	24	85	F	23	Failed	M	19months	109
18	M	Malignant schwannoma	19	35	114	F	31	Failed	F	3	127
									M	2	142
19	M	Hodgkin's disease	17	35	77	F	35	134	F	12	150
									F	10	96
20	F	Hodgkin's disease	17	33	146	M	33	95	F	3	149
									M	3	115
21	F	Hodgkin's disease	19	35	91	M	37	169	M	9	151
									M	5	No sample
									M	3	No sample
22	M	Wilms' tumour	1	31	130	F	36	89	F	2	109
23	M	Wilms' tumour	5	32	148	F	36	101	M	6	164
									M	13months	141

24	F	Malignant lymphoma	14	36	155	M	37	143	F	13	124
		Breast cancer [2]	33						M	9	146
25	F	Neuroblastoma	1	36	129	M	37	192	F	9	162
26	F	Hodgkin's disease	19	37	120	M	43	131	F	13	160
									M	10	162
27	F	Wilms' tumour	2	36	104	M	40	100	M	7	102
									F	5	76
28	F	Wilms' tumour	2	34	122	M	35	79	M	3	120
									M	3	163

[1] Offspring in birth order, [2] Secondary cancers.

Table 3.6: Characteristics of the trio and WRI validation study donor groups with mean and median chromatid aberration frequencies.

Donor group	Number	Male/female	Mean age[1] (years)	Median age[2] (years) (range)	Mean aberration frequency ± S.E.[3]	Median aberration frequency (range)	*P*[4]	*P*[5]
WRI validation	27	11/16	33.44	30 (20 – 54)	100.91 ± 3.28	95.43	0.058	-
Cancer survivor	23	8/15	33.13	34 (25 – 37)	118.35 ± 2.96	123.00	0.767	0.009
Partner	23	15/8	34.39	35 (26 – 43)	118.87 ± 3.02	114.00	-	0.058
Offspring	38	22/16	5.50	4.50 (4mths – 14)	131.58 ± 2.31	124.00	0.345	0.001
Offspring – 605	37	22/15	5.48	4 (4mths – 14)	124.16 ± 2.25	124.00	0.429	0.002

[1] Mean age at time of sampling, [2] Median age at time of sampling, [3] S.E.: Standard error, [4] Significance level achieved when comparing the median induced chromatid aberration frequency of each group to that of the partner control group, [5] Significance level achieved when comparing the median induced chromatid aberration frequency of each group to that of the WRI validation study group.

Table 3.7: Comparison of the radiosensitivity profiles between the WRI validation study group, childhood and adolescent cancer survivors, their partner controls and offspring.

Donor group	N[1]	%	*P*[2]	N[3]	%	*P*[4]
WRI validation	0	0.00	0.090	3	11.11	-
Cancer survivor	0	0.00	0.233	12	52.17	0.002
Partner	3	13.04	-	8	34.78	0.084
Offspring	7	18.42	0.729	20	52.63	<0.001

[1] Number of individuals displaying elevated radiosensitivity using the 90th percentile cut-off point of the partner control group, [2] Significance level achieved when comparing % of radiosensitive donors to the partner control group, [3] Number of individuals displaying elevated radiosensitivity using the 90th percentile cut-off point of the WRI validation study group, [4] Significance level achieved when comparing % of radiosensitive donors to the WRI validation study group.

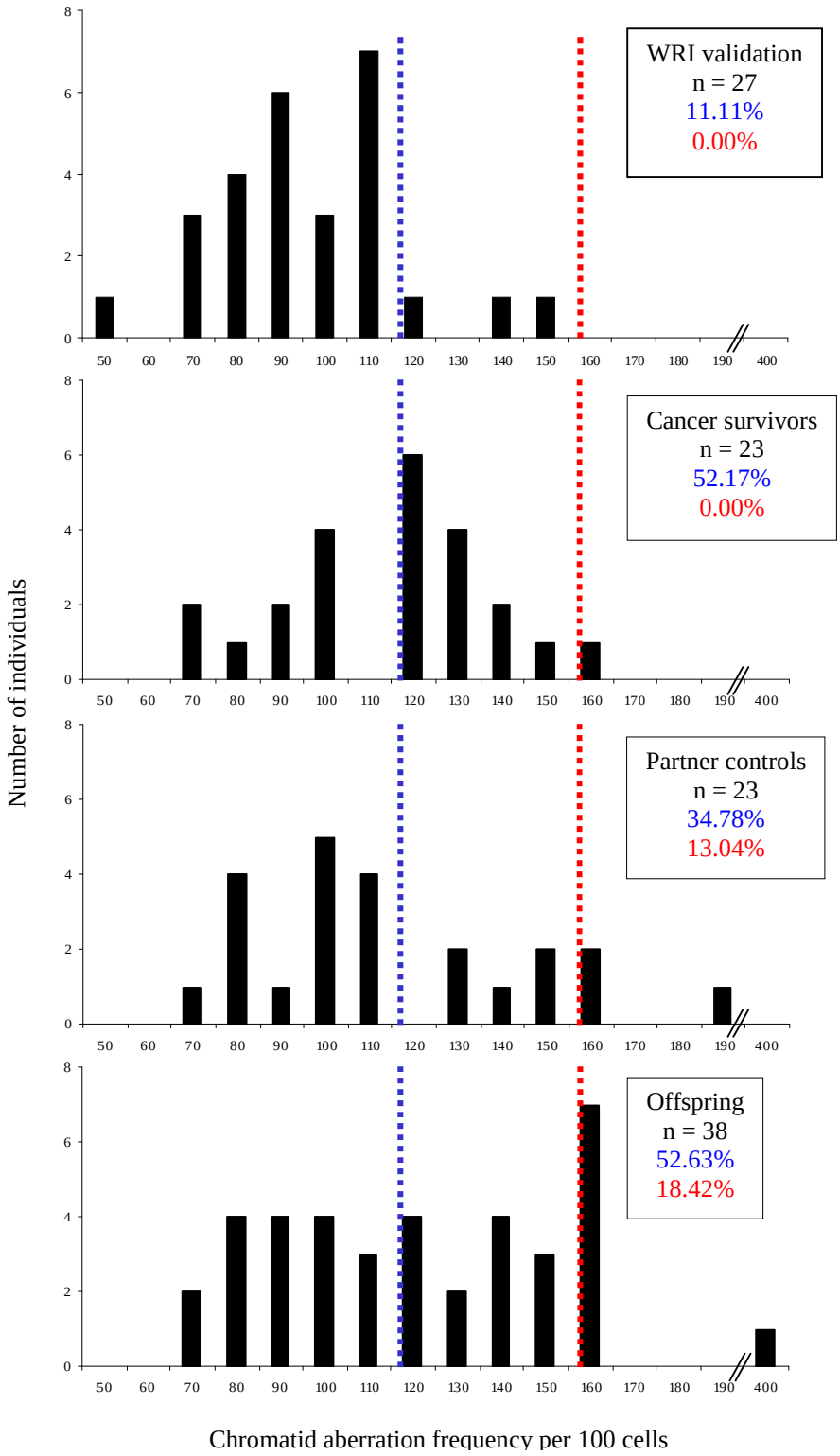


Figure 3.2: Distributions of chromatid aberration frequencies in the WRI validation study group, the survivors of childhood and adolescent cancer, their partners and offspring. The dotted blue vertical line represents the cut-off point for a radiosensitive response (122.6 chromatid aberrations per 100 cells), based on the 90th percentile of the WRI validation study group and the dotted red vertical line represents the cut-off point for a radiosensitive response (162.4 chromatid aberrations per 100 cells), based on the 90th percentile of the partner control group. Text in blue and red represent the proportions of radiosensitive individuals based on the WRI validation study group and partner control group cut-off points, respectively.

Heritability of radiosensitivity

Since G_2 radiosensitivity of the offspring group could potentially be correlated to that of both parents (presuming it is under genetic control), the data were also analysed by examining the sensitivity profiles of offspring in relation to their parents. This is illustrated in Figure 3.3. Out of a group of 38 offspring, 20 individuals displayed the sensitive phenotype as determined by the 90^{th} percentile cut-off point of the WRI validation control group. Further examination of the radiosensitivity profiles of the parents of this group, revealed that 80% of these offspring (16/20) had one parent who displayed a sensitive and one who displayed a normal response, whilst 15% (3/20) had parents who both displayed a sensitive response and only 5% (1/20) had parents that both displayed normal response. Similarly, examination of the sensitivity profiles of the parents of the remaining 18 children who displayed a non-radiosensitive phenotype using the cut-off point of 122.6 chromatid aberrations per 100 cells, revealed that 61.11% (11/18) had one parent who displayed a sensitive and one who displayed a normal response, whilst 38.89% (7/18) had parents that both displayed normal response and none of the offspring had parents who both displayed a sensitive response. A statistical significance was observed between the number of radiosensitive and non-radiosensitive offspring who had both normal parents ($P = 0.016$), although this was not the case for offspring with one normal and one high parent ($P = 0.288$), or for offspring with both parents displaying the radiosensitive phenotype ($P = 0.232$).

Heritability of the radiosensitivity phenotype in the Danish families was investigated further using a more sophisticated statistical analysis undertaken by Professor Ranajit Chakraborty and is reported in Curwen *et al.* (2005). In summary, the analysis suggested a major gene locus at which the dominant allele (labelled as *A*) conferred a genotypic mean

induced frequency of 143.53 chromatid aberrations per 100 cells (same for *AA* and *AB* genotypes), with the recessive homozygotes (*BB*) having a mean of 97.82 induced chromatid aberrations per 100 cells. With frequencies of 0.450 and 0.550, for the dominant and recessive phenotypes respectively, the genetic variance attributable to the putative locus was 517.13, with residual variance within each genotype being 250.94. Thus, the estimate of the variance of chromosomal radiosensitivity attributable to the putative dominant major gene locus was: 513.13 / (513.13 + 250.94) = 0.673, suggesting that more than two-thirds of the phenotypic variance of chromosomal radiosensitivity is attributable to a putative major gene locus (with dominant effect). This estimate is also consistent with an alternative prediction performed by SOLAR software (Almasy and Blangero 1998). For the unadjusted induced G_2 chromatid aberration frequency, the heritability estimate by this variance component method was 0.607 ± 0.215 (significantly different from zero, $P = 0.006$).

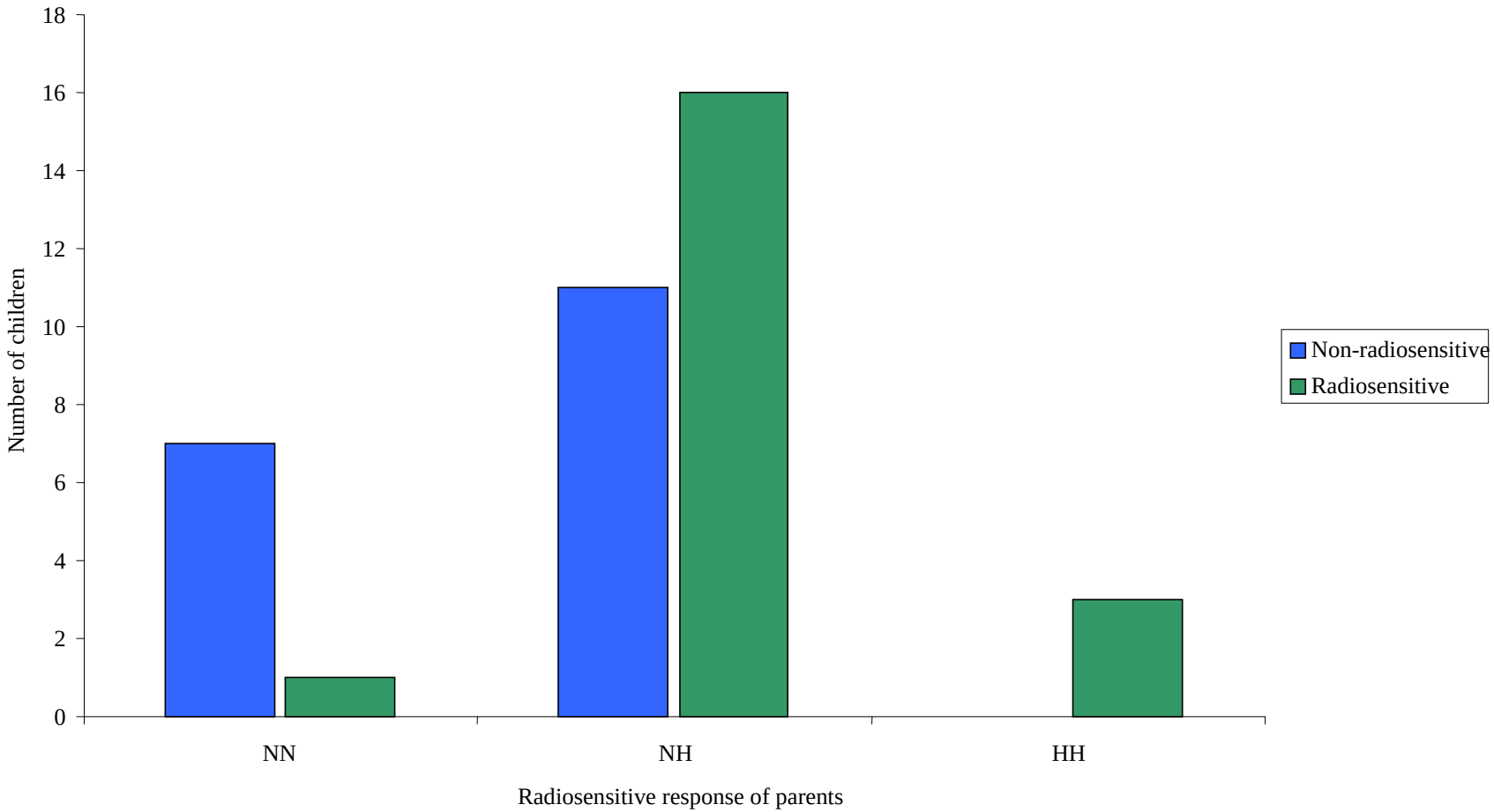


Figure 3.3: Chart to illustrate the number of children with a radiosensitive/non-radiosensitive response (determined by the 90th percentile cut-off point of the WRI validation study group of 122.6 chromatid aberrations per 100 cells) in comparison to the response of their parents. NN: both parents having a normal response, NH: one parent with a normal and one with a high response, HH: both parents displaying a high response.

Assessing mitotic inhibition in cancer survivors, their partners and offspring

The degree of mitotic inhibition after exposure of lymphocytes to 0.5 Gy X-rays was examined in the survivors of childhood and adolescent cancer, their partners and offspring. Mean and median mitotic indices in unirradiated cells were determined for each group of donors (refer to Table 3.8). Comparison of mean mitotic indices highlighted similarities between the offspring and partner control groups but that achieved for the cancer survivor group was slightly lower. However, analysis of variance did not demonstrate any statistically significant difference between the groups ($P = 0.293$). In addition, comparison of median mitotic indices revealed no statistically significant differences between the cancer survivors ($P = 0.194$) or offspring ($P = 0.623$) and the partner control group (refer to Figure 3.4). Similarly, for mitotic inhibition, analysis of variance of the three donor groups revealed no statistically significant difference ($P = 0.212$) and comparison of median values obtained for mitotic inhibition also revealed no statistically significant differences between the cancer survivors ($P = 0.660$) or offspring ($P = 0.171$) and the partner control group (refer to Figure 3.5). There were no significant relationships observed between mitotic index and chromatid aberration frequency (refer to Figure 3.6 and Table 3.9), nor mitotic inhibition and chromatid aberration frequency (refer to Figure 3.7 and Table 3.9), in any of the donor groups.

Table 3.8: Mitotic indices and mitotic inhibition in childhood and adolescent cancer survivors, their partners and offspring.

Endpoint	Donor group	n	Mean ± sd[1]	CV[2] (%)	*P*[3]	Median (range)	*P*[4]
Mitotic index[5] (%)	Survivors	23	1.47 ± 0.77	52.49		1.40 (0.30 – 3.20)	0.194
	Partners	23	1.83 ± 0.90	49.04	0.293	1.80 (0.10 – 3.00)	-
	Offspring	38	1.86 ± 1.13	60.67		1.60 (0.40 – 4.90)	0.623
Mitotic inhibition (%)	Survivors	23	79.73 ± 17.90	22.45		84.62 (33.3 – 100.00)	0.660
	Partners	23	77.81 ± 20.31	26.10	0.212	82.35 (0.00 – 92.31)	-
	Offspring	38	84.82 ± 11.01	12.98		86.19 (55.56 – 100.00)	0.171

[1] Standard deviation, [2] Coefficient of variation, [3] Significance level achieved for one-way analysis of variance, [4] Significance level achieved when comparing the mitotic index and degree of mitotic inhibition achieved in each group to that of the partner control group, [5] Mitotic index in unirradiated cells.

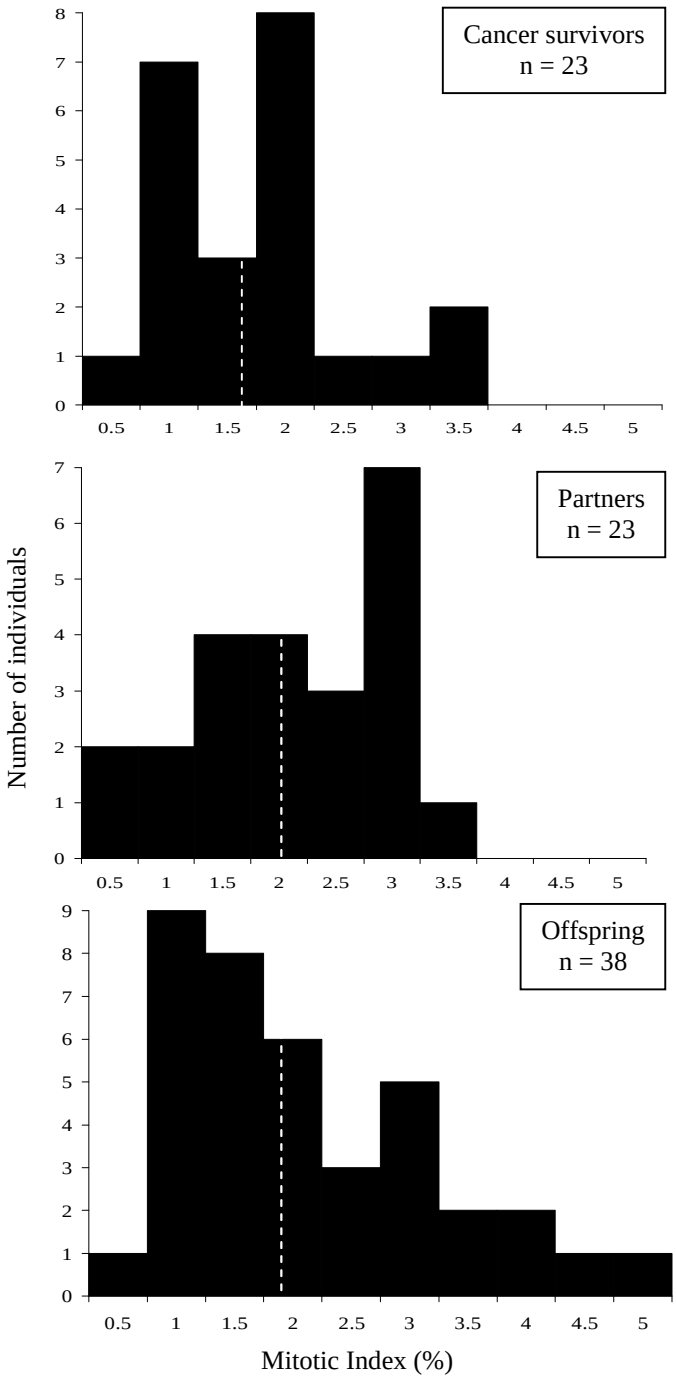


Figure 3.4: Chart showing mitotic indices in unirradiated cells of childhood and adolescent cancer survivors, their partners and offspring. Vertical dashed lines indicate medians. Statistical significances of comparisons are given in Table 3.8.

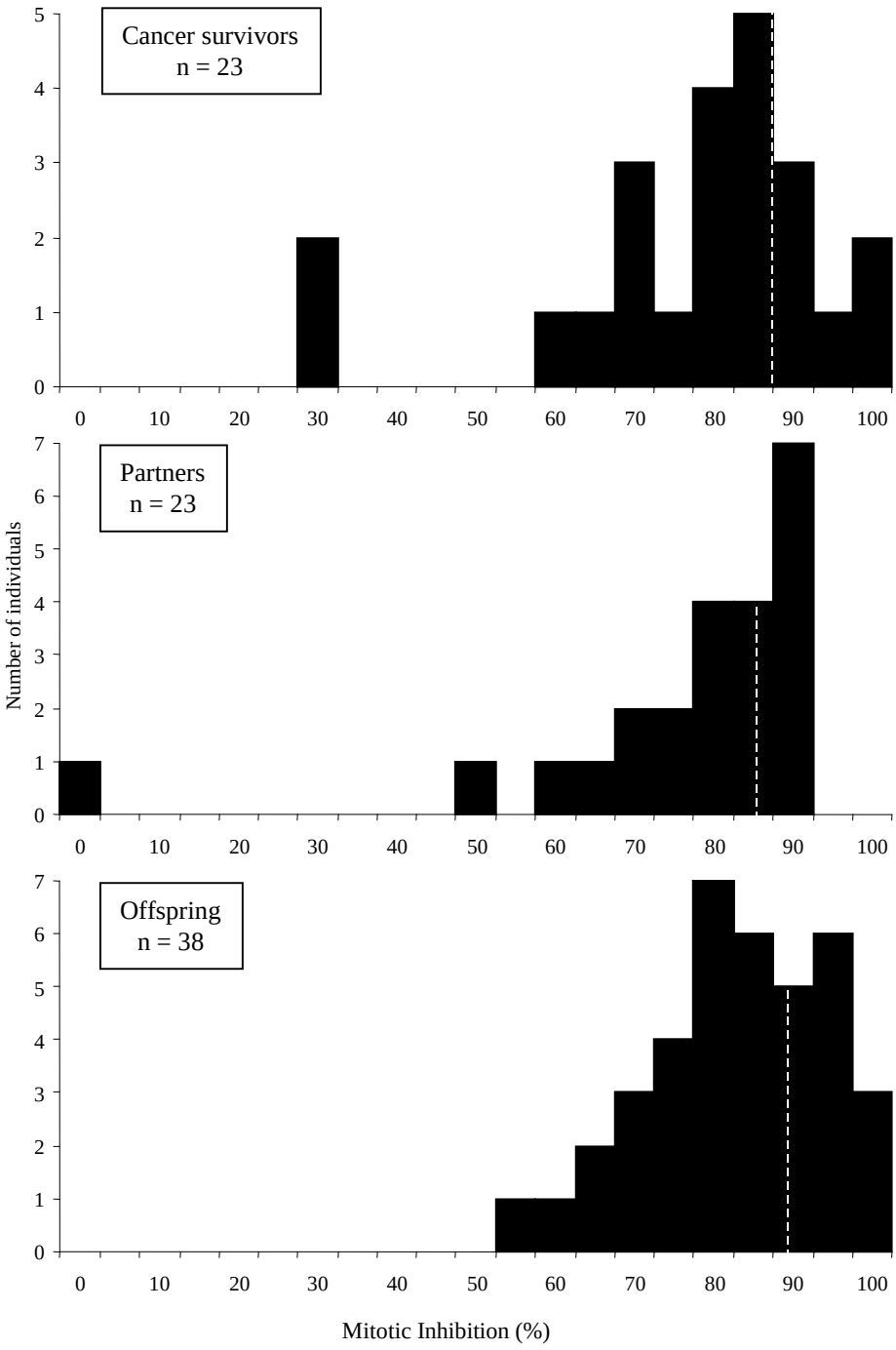


Figure 3.5: Chart showing mitotic inhibition in irradiated blood cultures from childhood and adolescent cancer survivors, their partners and offspring. Vertical dashed lines indicate medians. Statistical significances of comparisons are given in Table 3.8.

Table 3.9: Relationships of mitotic index and mitotic inhibition with chromatid aberration yields in survivors of childhood and adolescent cancer, their partners and offspring.

Donor group	Endpoint	Spearmans correlation	
		r[1]	*P*[2]
Cancer survivor	Mitotic index[3]	0.026	0.906
	Mitotic inhibition	0.045	0.837
Partner controls	Mitotic index	0.298	0.167
	Mitotic inhibition	0.182	0.405
Offspring	Mitotic index	0.002	0.989
	Mitotic inhibition	0.042	0.802

[1] Correlation coefficient, [2] Significance level achieved when using Spearmans rank correlation analysis, [3] Mitotic index in unirradiated cells.

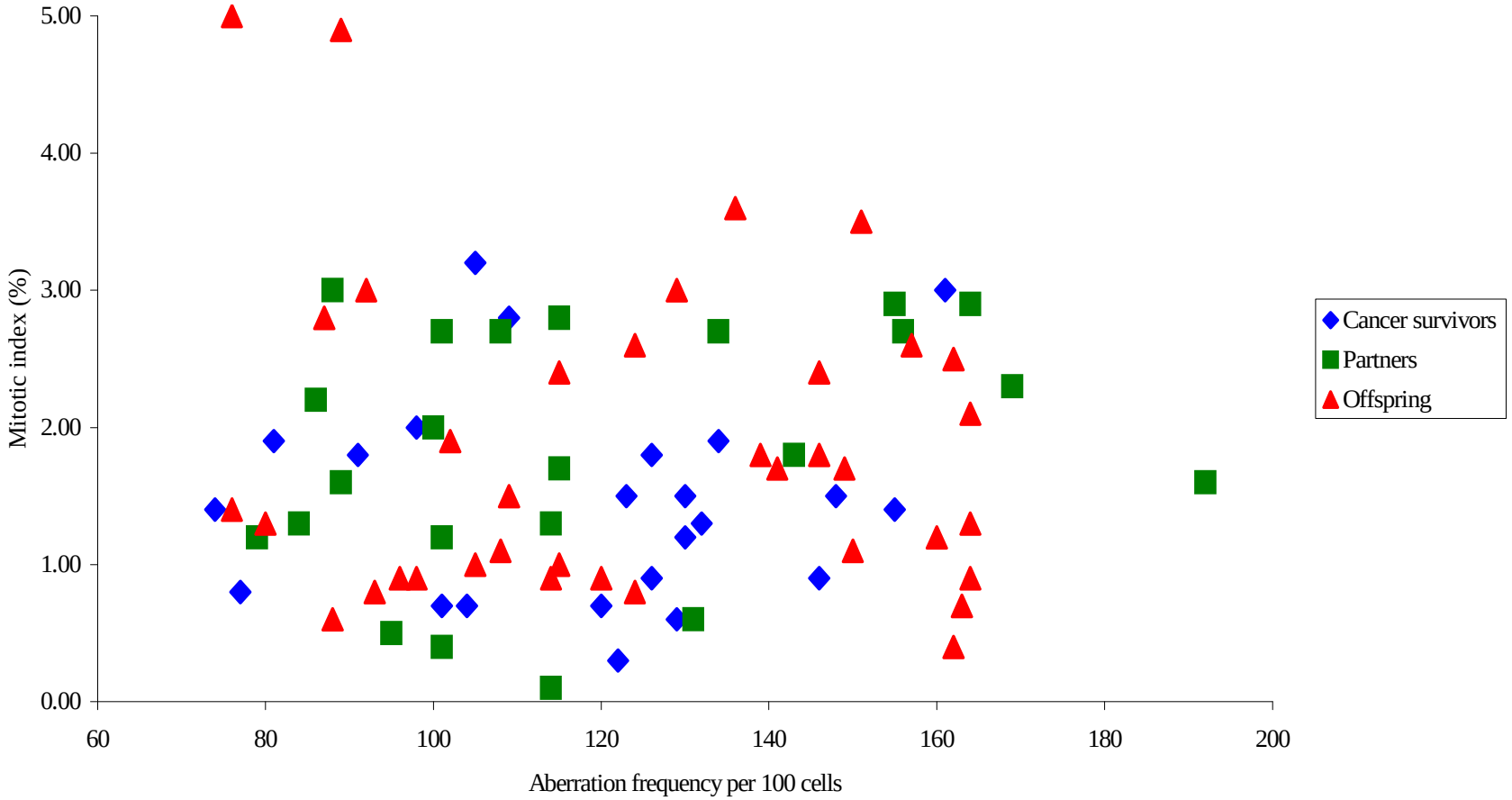


Figure 3.6: Correlation between mitotic index in unirradiated cells and chromatid aberration frequencies for childhood and adolescent cancer survivors, their partners and offspring.

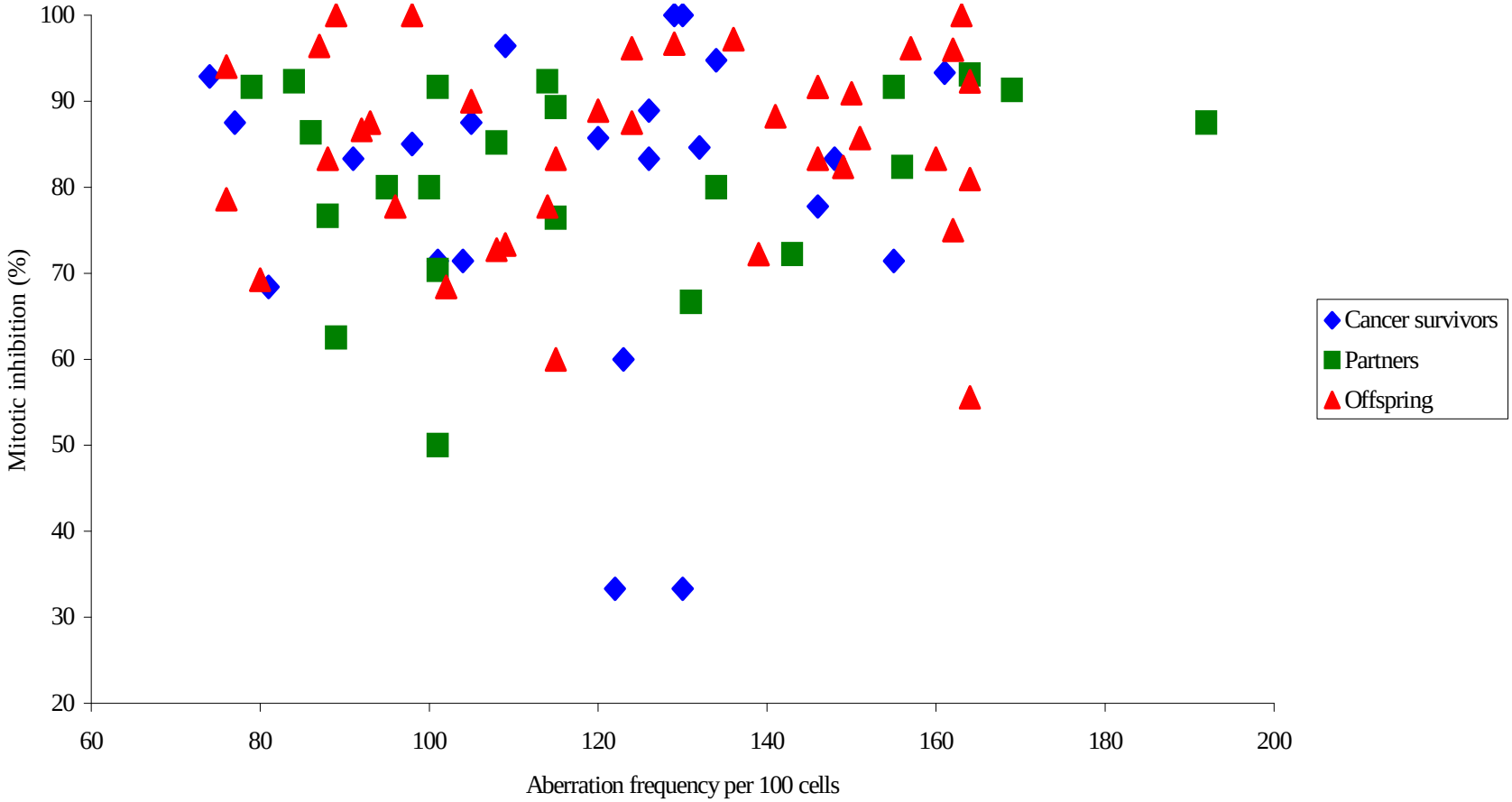


Figure 3.7: Correlation between mitotic inhibition and chromatid aberration frequencies for childhood and adolescent cancer survivors, their partners and offspring.

Discussion

G_2 assay control measures

Since it has previously been reported that the G_2 assay requires stringent technical conditions to obtain reproducible results (Bryant *et al.*, 2002; Scott *et al.*, 1996), and problems associated with storage conditions and transportation of blood samples over long distances have also been noted (Scott *et al.*, 1999), appropriate control measures were taken in this study to monitor these effects. Assay reproducibility was initially confirmed in the WRI laboratory as documented in Chapter Two. The subsequent analyses of samples taken periodically from the internal assay control and set up for culture in conjunction with samples received from Denmark, were used for the continuous the assessment of assay reproducibility and no problems were observed. Sample storage and transportation conditions were also investigated in the WRI laboratory, as documented in Chapter Two. Again, reproducibility was not shown to be an issue for samples transported over short distances and stored in varying conditions. Nevertheless, samples were sent to WRI from Denmark in secondary packaging, at ambient temperature and in the possession of a personal courier at all times. In this way, samples were not subjected to significant temperature fluctuations, which have been suggested as a possible cause of intra-individual variation (Bryant *et al.*, 2002) and any potential effect of transportation was thus minimised. In addition, any potential variation that could be associated with transport was monitored by the inclusion of a sample from a Danish control with each shipment received. Significant intra-individual variation was observed in the samples received from this individual. However, this volunteer became pregnant during the study and was pregnant from shipment six through to ten. Further analysis revealed that the first five samples provided gave no indication of intra-individual variability but the final four did

produce variable results. Hence, the variability observed could be due to pregnancy related hormonal changes. Evidence to strengthen this theory comes from several previous studies. Roberts *et al.* (1997) reported progesterone to have a profound effect upon the radiosensitivity of females, as measured by the induction of chromosomal aberrations *in vitro*. Kanda & Hayata (1999) examined the *in vitro* effect of estradiol on chromosome aberrations in fresh blood samples from a healthy female and demonstrated a significantly higher yield with the addition of the hormone. Ricoul *et al.* (1997) reported increased sensitivity to ionising radiation in blood samples from pregnant women when compared to those from non-pregnant women, as measured by chromosome breakage. Furthermore, i*n vivo* studies in pregnant mice suggested a direct relationship between increases of chromosomal breakage and gestational hormones (Ricoul & Dutrillaux, 1991).

Radiosensitivity in childhood and adolescent cancer survivors, their partners and offspring

Previous studies have suggested that G_2 chromosomal radiosensitivity could be a marker of low-penetrance cancer predisposition genes (Roberts *et al.*, 1999; Scott, 2000; Scott *et al.*, 1994a). In this study, the survivors of childhood and adolescent cancer, their partners and offspring were analysed for chromatid aberrations using the G_2 assay and their radiosensitivity profiles determined, using the 90^{th} percentile of the control group of partners as the cut-off point. Results indicated no statistically significant differences between the three groups in terms of sensitivity and thus, an association between increased G_2 chromosomal radiosensitivity and predisposition to childhood/adolescent cancer could not be demonstrated. However, when using the 90^{th} percentile of the WRI validation study group as the cut-off point for a sensitive response, a clear distinction in radiosensitivity profiles was observed for both the childhood and adolescent cancer survivors and their

offspring, whilst nothing significant was observed for the partner control group (refer to Figure 3.2). On this basis, the results appear to confirm the previous findings of Baria *et al.* (2002) who investigated G_2 chromosomal radiosensitivity in young cancer patients and suggested that a substantial proportion of early onset cancers are associated with the inheritance of predisposing genes of low penetrance involved in coping with damage to the genome.

With the results in mind, it is important to ask the question as to whether the partner controls were a suitable control group. Besides the fact that a transportation effect could not be entirely ruled out for the enhanced sensitivity profiles observed in all three family groups, this question proved difficult to explain. Nothing was obviously apparent and there were no significant differences in either the median level of chromatid aberrations or the percentage of individuals classed as radiosensitive between each group.

One possible explanation could be that the partners of the childhood and adolescent cancer survivors themselves form a distinct group with a heightened radiosensitivity profile. Although based purely on speculation, cancer survivors may be more likely to attract or accept partners with some knowledge of cancer because the partners have experienced it within their own families. Thus, the partner control group may have been comprised of a greater proportion of individuals with cancer predisposition than a control group taken randomly from a healthy population. Regardless of this, a thorough check of family questionnaires and cancer registries confirmed that none of the partners themselves had suffered from cancer.

Another simple explanation could be the very low numbers of samples. Increasing the numbers of families enlisted to the project may help to further address the issue of predisposition to cancer mediated through low penetrance genes, as well as inherited susceptibility. Moreover, recruitment and exclusion of specific cancer types may be beneficial. Early onset cancer is a common feature of inherited susceptibility. Ideally, the present study would have involved testing many more patients with specific early onset cancer types. In reality, the study consisted of a heterogeneous mix of cancers, including 10 cases of Hodgkin's disease, six cases of Wilms' tumour, two cases of neuroblastoma and one each of rhabdomyosarcoma, teratoma, lymphoepithelioma, pineocytoma and malignant lymphoma. Wilms' tumour, a paediatric malignancy of the kidney, is known to have an inherited component. Wilms' tumour occurs sporadically in the majority of cases, but can also be due to a constitutive mutation in the highly penetrant cancer susceptibility gene *WT1*. Up to 10% of cases are bilateral (occurring in both kidneys), suggesting an underlying genetic predisposition. Between 1 and 2% of bilateral cases are believed to occur as the result of familial transmission, the remainder occurring due to a *de novo* mutation (Marsh & Zori, 2002). In this study, three of the six cases of Wilms' tumour displayed enhanced sensitivity when using the 90^{th} percentile of the WRI validation study group as the cut-off point. Further investigation of the medical records of these individuals revealed that all six cases were unilateral (occurring in one kidney) and there was no recorded family history of Wilms' tumour for any, but since the *WT1* gene was not identified until the late 1970's, genetic testing was not undertaken. The example of Wilms' tumour illustrates the importance of inclusion criteria and medical records in such a study. In addition, WT1 functions as a zinc finger transcription factor to repress the number of growth promoting genes via DNA binding and plays no known role in the numerous DNA damage repair processes. Likewise, of the ten cases of Hodgkin's disease

reported in this study, four exhibited evidence of enhanced radiosensitivity using the cut-off point defined by the WRI validation study group. Similar results were reported by Baria *et al.* (2002) who found that three out of six young cancer patients with Hodgkin's disease displayed enhanced radiosensitivity.

Recent literature, based on the data in the nationwide Swedish Family-Cancer Database, has suggested that cancer is mainly an environmental disease, with a minor heritable aetiology (Hemminki & Bermejo, 2005; Hemminki *et al.*, 2005) and quoted cancers in offspring such as Hodgkin's disease, as well as cancers of the nervous system, testis and kidney as having only 0.7, 1.8, 0.5 and 2.8% familial proportion (i.e. % of affected offspring with affected parent), respectively. Furthermore, Lichtenstein *et al.* (2000) studied nearly 45,000 pairs of monozygotic and dizygotic twins to establish the relative contribution of environmental and hereditary factors on various cancer incidence and demonstrated no concordance between twins for cancers of the kidney, thyroid, bone and Hodgkin's disease. High concordance was demonstrated among twins with stomach, colorectal, lung, breast and prostate cancer. Lichtenstein *et al.* (2000) concluded that inherited genetic factors make only a minor contribution to susceptibility to most types of neoplasms. Furthermore, a study by Mack *et al.* (1995) suggested that in young adulthood, Hodgkin's disease is initiated by an environmental exposure, possibly to an infectious agent, namely the Epstein-Barr virus (EBV). Nevertheless, monozygotic twins of patients with Hodgkin's disease had a greatly increased risk of Hodgkin's disease, suggesting the disease does occur preferentially in certain families and may be hereditary. However, of the pairs of twins concordant for the disease, nodular sclerosing Hodgkin's disease accounted for the majority of the diagnoses suggesting that this subgroup only was genetically susceptible. Nodular sclerosing Hodgkin's disease is a distinct histological

subtype, with a low prevalence of EBV genome in the tumour, indicating a fundamental genetic influence on the pathophysiology. Immune abnormality associated with certain human leukocyte antigen haplotypes and abnormal cytokine production in affected patients and close relatives of some patients is associated with this sub-type and thus, genetic susceptibility is most likely to result from immune system functionality. With this in mind, careful consideration of the childhood and adolescent cancer types to be studied should be given when extending the current study. However, since early onset cancer is comparatively rare, accounting for less than 2% of all cancers diagnosed in patients of less than 20 years in the UK (Baria *et al.*, 2002), concentration on specific groups or subgroups for radiosensitivity studies could prove difficult.

Heritability of radiosensitivity

Whatever the reasons for increased sensitivity in all three family groups when using the partner controls as the cut-off point to measure radiosensitivity, this has resulted in doubts as to the relevance of the G_2 assay as a measure of predisposition to cancer. Nevertheless, in comparison with other studies, evidence of heritability of the radiosensitivity phenotype has been demonstrated here. The studies of Roberts *et al.* (1999) and Scott (2000) provided the initial evidence for heritability of the G_2 chromosomal radiosensitivity phenotype, through examination of families of patients with breast cancer. First-degree relatives of patients demonstrating enhanced sensitivity were found more likely to be radiosensitive than first-degree relatives of patients with a normal response. Segregation analysis of 95 family members suggested that 82% of the variability in G_2 radiosensitivity could be accounted for by a single major gene, with two alleles combining in an additive manner to give complete heterozygote expression. However, although this model explained the majority of the patterns of segregation observed in the breast cancer families,

there were a few families for which the inclusion of a second rarer gene with a similar additive effect on radiosensitivity gave a better fit to the data. Segregation analysis reported in this thesis, performed by Professor Chakraborty, found that the one-locus model was the most suitable for the data, with alleles having a dominant-recessive relationship with regard to effect on the induced G_2 chromatid aberration yield. In addition, the contribution of this locus to the total variance of the G_2 chromatid aberration yield reported here appears to be lower than that estimated by Roberts *et al.* (1999), although due to limited sample sizes, this difference may not be real. Besides this, there is agreement between the two studies on the involvement of a single major gene locus, which accounts for over two-thirds of the variation of G_2 sensitivity and the data reported here is supportive of genetic heritability of radiosensitivity in families of unselected cancer cases.

G_2 checkpoint control activity

The first demonstration of a G_2 checkpoint defect following ionising radiation exposure in human cells came from observations of AT patients (Scott & Zampetti-Bosseler, 1982; Zampetti-Bosseler & Scott, 1981). These studies found that the typical arrest of G_2 cells following exposure to ionising radiation was much less pronounced in these individuals than in normal cells. From this it was suggested that a G_2 checkpoint defect might account for the enhanced chromosomal radiosensitivity of cells from AT patients, due to less time being available for repair of DNA damage before the onset of mitosis. Similarly, a study by Scott *et al.* (2003) proposed that less G_2 arrest (measured as the degree of mitotic inhibition) in irradiated cells of breast cancer patients than in healthy female controls suggesting that this might be a contributory factor to their enhanced chromosomal radiosensitivity. Nevertheless, on further examination of the results a number of observations put the hypothesis into doubt. Firstly, the degree of mitotic inhibition

observed was significantly greater in female than male controls. However, this was not accompanied by a difference in G_2 chromosomal radiosensitivity between the sexes. Secondly, an inverse correlation between the extent of inhibition and age was observed in controls, i.e mitotic inhibition showed a significant decline with age, but no age related increase in radiosensitivity was observed. With this in mind, Scott and his colleagues used the female normals for comparison with patients and thus observed significantly less mitotic inhibition in the patients than in normal females. A weak inverse correlation between the extent of mitotic inhibition and the amount of chromosome damage was also observed, this being in all females (patients and controls grouped). To determine if this was the case in the present study, the efficacy of the G_2 checkpoint control was examined by monitoring the degree of mitotic inhibition in the three family groups, following exposure of cells to X-rays. No differences between mean or median values were revealed for both mitotic indices and the degree of mitotic inhibition observed between the groups. In addition, no relationships were observed between mitotic indices and yields of chromatid aberration frequencies or the degree of mitotic inhibition and yields of chromatid aberration frequencies. In conclusion, the present study could not provide confirmation of a relationship between G_2 checkpoint control efficacy and enhanced G_2 chromosomal radiosensitivity.

General conclusions

It must be stressed that if reliance had been placed solely on the in-house WRI validation study group for comparison with the childhood and adolescent cancer survivors and their offspring, then confirmation of an association between G_2 radiosensitivity and cancer susceptibility would not be in doubt. However, there is no obvious plausible reason for why the partner control group were statistically indistinguishable from the childhood and

adolescent cancer survivor and offspring groups, and thus it must be concluded that a relationship between cancer predisposition, G_2 chromosomal radiosensitivity and heritability of the phenotype has not been elucidated. The work on G_2 chromosomal radiosensitivity has been reported in Curwen *et al.* (2005). Future studies will expand all three family groups and also investigate further whether a transport factor could be influencing the results.

CHAPTER 4

Investigating the association of G_2 chromosomal radiosensitivity with DNA repair gene polymorphisms in the survivors of childhood and adolescent cancer, their partners and offspring.

Introduction

Variation in radiosensitivity is influenced by genetic factors. However, the exact genetic mechanisms underlying inter-individual differences remain unknown although control is likely to be dependent on the combined effect of several different pathways. The importance of the role of DNA repair in the protection of the genome and prevention of cancer is however appreciated. Since G_2 chromosomal radiosensitivity indirectly measures the ability of cells to repair DNA damage induced by ionising radiation exposure, variants in DNA damage recognition and repair genes may explain the inter-individual variation observed and thus have been examined in this study.

Materials and methods

A panel of single nucleotide polymorphisms (SNPs) were investigated in the Danish trio population consisting of *ADPRT* t2444c, *APEX* t649g, *hOGG1* c1285g, *hOGG1* g445a, *XPD* g935a, *XPD* a2251c, *XRCC1* g1301a, *XRCC1* c685t, *XRCC1* g944a, *XRCC2* c139t, *XRCC3* c1075t, *XRCC4* t401c, *XRCC4* g921t, *XRCC1* microsatellite (a $[AC]_n$ repeat region in the 3' untranslated region), *XRCC3* microsatellite (a $[AC]_n$ repeat region located

in intron 3) and *XRCC5* microsatellite (a $[GAPyA]_n$ repeat region located at 120kb 5'). Table 4.1 provides further information on the SNPs analysed.

The SNPs investigated were essentially analysed using two methods: PCR followed by restriction fragment length polymorphism (RFLP) analysis with genotypes being determined by either agarose gel electrophoresis or genotyping on an ABI Prism 310 platform; real-time PCR and allelic discrimination using an ABI Prism 7000 plate reader (refer to Figure 4.1). Microsatellite sizing was undertaken via a multiplex PCR using fluorescently labelled primers followed by size discrimination of PCR products on an ABI Prism 310 platform. Prior to SNP analysis, biological paternity and maternity testing was performed on the trio DNA samples by investigating four minisatellite loci with a variable number of tandem repeats and a low background mutation rate, as described in Rees *et al.* (2006).

For quality control purposes, positive and negative controls were used in all assays. Positive controls were DNA samples from a newborn cohort that had previously been genotyped for the same range of SNPs and in most cases, samples with one mutant and one wild type allele were chosen. Distilled water was used to act as the non-template negative control. Genotyping was undertaken blind and 10% of all samples were repeated. On completion of genotyping of the cohort, all assigned genotypes were checked and 10% checked again by an independent third party.

Table 4.1: Details of the single nucleotide polymorphisms investigated.

Gene	Base change	Position	Amino acid change	dbSNP reference	Repair pathway	Method reference
ADPRT	T > C	2444	Val762Ala	rs17853760	BER[1]	Cottet *et al.*, (2000)
APEX	T > G	649	Asp148Glu	rs17858508	BER	Hu *et al.*, (2001)
hOGG1	C > G	1285	Ser326Cys	rs1052133	BER	Wikman *et al.*, (2000)
hOGG1	G > A	445	Arg46Gln	n/a	BER	Wikman *et al.*, (2000)
XPD	G > A	935	Asp312Asn	rs1799793	NER[2]	Lunn *et al.*, (2000)
XPD	A > C	2251	Lys751Gln	rs13181	NER	Spitz *et al.*, (2001)
XRCC1	G > A	1301	Arg399Gln	rs25487	BER	Lunn *et al.*, (2000)
XRCC1	C > T	685	Arg194Trp	rs1799782	BER	Lunn *et al.*, (2000)
XRCC1	G > A	944	Arg280His	rs25489	BER	Lunn *et al.*, (2000)
XRCC1	Microsatellite	-	Non-coding	-	BER	Price *et al.*, (1997)
XRCC2	C > T	139	Pro36Ser	rs1871892	DSBR[3] (HR)[4]	n/a
XRCC3	C > T	1075	Thr241Met	rs861539	DSBR (HR)	Shen *et al.*, (1998)
XRCC3	Microsatellite	-	Non-coding	-	DSBR (HR)	Price *et al.*, (1997)
XRCC4	T > C	401	Ile134Thr	rs28360135	DSBR (NHEJ)[5]	Ford *et al.*, (2000)
XRCC4	G > T	921	Ser307Ser	rs1056503	DSBR (NHEJ)	Ford *et al.*, (2000)
XRCC5	Microsatellite	-	Non-coding	-	DSBR (NHEJ)	Price *et al.*, (1997)

[1]Base excision repair; [2]Nucleotide excision repair; [3]Double-strand break repair; [4]Homologous recombination; [5]Non-homologous end-joining.

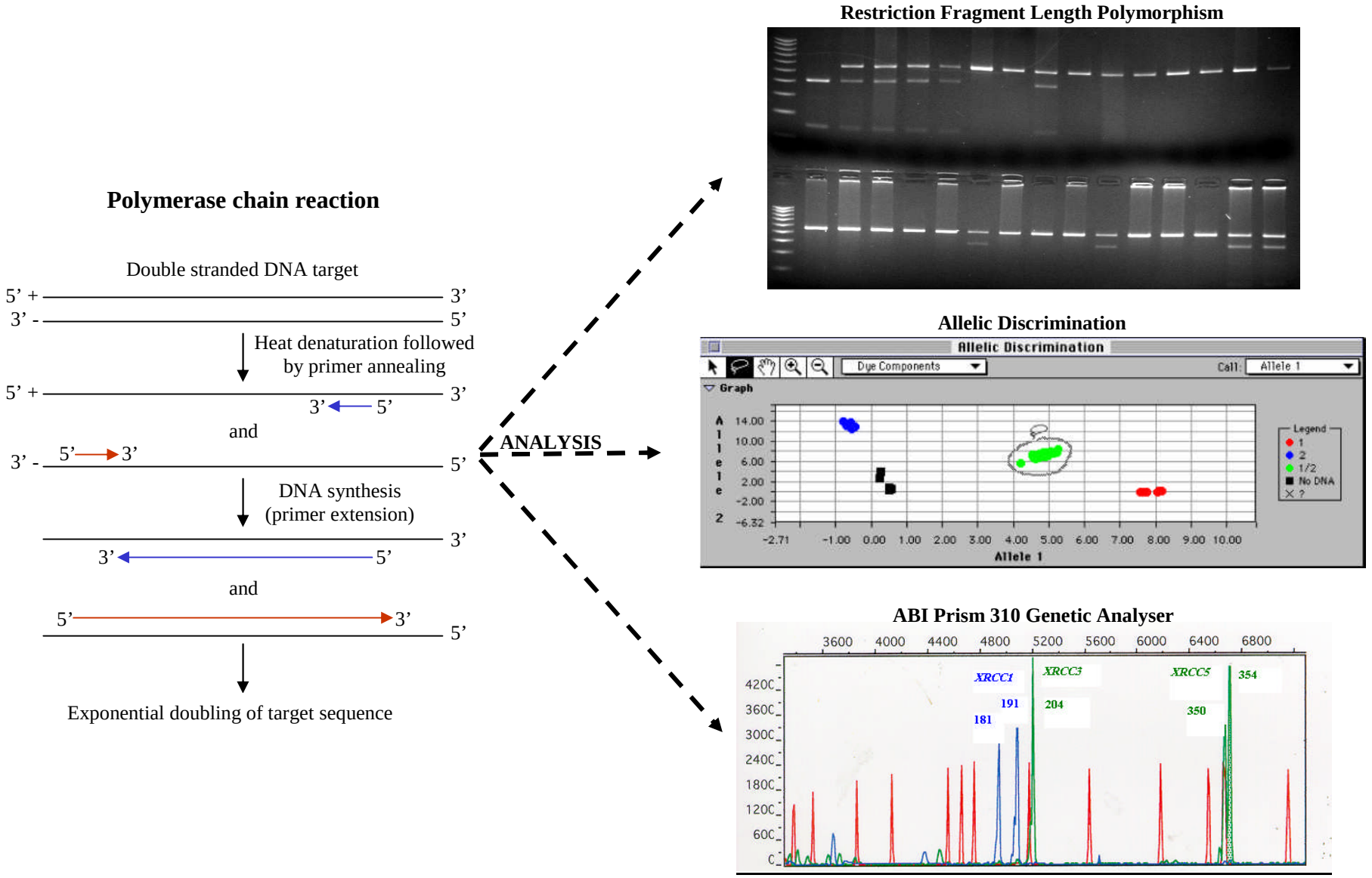


Figure 4.1: Methods used to examine DNA repair gene polymorphisms.

Restriction fragment length polymorphism analysis

PCR primer design

For each SNP analysed, primers flanking the DNA region of interest region were used. Most primer sequences were previously published in the literature and thus were designed using the reported sequences. All primers were manufactured by MWG-Biotech Ltd (with the exception of the primers for *XRCC2* c139t which were designed independently by Applied Biosystems and the SNP analysed using the real-time PCR/Taqman SNP genotyping assay). Primers were purified by HPLC (high performance liquid chromatography) and provided lyophilised. Table 4.2 provides the sequences and corresponding melting temperatures for the forward and reverse primers used. Upon arrival at WRI, primers were hydrated to a 100pmol/µl stock concentration by the addition of an appropriate quantity of sterile water (Braun, Ref 3627608) and then diluted 1:4 to a 25pmol/µl working concentration by mixing 25µl stock solution with 75µl sterile water. Primer stocks were stored at -20°C and the working solution at 4^{o}C. Primers for the *XRCC1, XRCC3* and *XRCC5* microsatellites were hydrated to a 50pmol/µl stock solution using sterile water and a combined 10x working primer mix containing 2pmol/µl of each primer prepared and stored in 70µl aliquots. Details are provided in Table 4.3. Stock solutions for the microsatellite primers and the 10x working primer mix were both stored at -20^{o}C.

PCR set up

With the exception of the real-time PCR method, all PCR protocols were initially optimised for use and standard operation procedures were followed. Before PCR set up the appropriate number of 0.2ml microamp capped PCR tubes (Applied Biosystems, Ref. N8010540) were labelled, including two tubes for the positive and negative controls.

Three batches of PCR reactions were performed for each polymorphism in order to genotype the entire trio cohort. The initial reaction consisted of families 1-13, the second reaction families 14-26 and the final reaction families 27 and 28 plus 10% of the samples repeated for quality control purposes. In most instances the repeats were samples 101, 201, 301, 401, 501, 601, 701, 801, 901 and 1001.

Initially, laboratory bench tops were swabbed with 70% ethanol (Fisher Scientific, Ref. E/0650/17) to reduce contamination. A specific mastermix of PCR reagents was prepared in a 1.5ml eppendorf tube (Anachem, Ref. 96-7514-6-01) for each SNP analysed, with the volume required determined by multiplying the amount for one tube by the number of reaction tubes and adding 10% to each total to ensure an adequate supply. Recipes for the mastermixes are detailed in Table 4.4. PCR buffers used in the mastermixes were obtained from ABgene: 10x Buffer I (Ref. AB-0242), 10x Buffer IV (Ref. AB-0289) and 10x Buffer V (Ref. AB-0290). To prepare the 2mM dNTP solution used, 100mM stocks of dATP, dTTP, dCTP and dGTP provided by ABgene (Ref. AB-0315) were diluted to 2mM dNTP working solution by the addition of 20µl each of 100mM stocks of dATP, dTTP, dCTP, dGTP to 920µl sterile water (Braun, Ref. 3627608). The solution was then vortexed to mix and stored at -20°C in 500µl aliquots. The $MgCl_2$ used in the mastermix solutions was also provided by ABgene at a concentration of 25mM and the *Taq* DNA polymerase (ABgene, Ref. AB-0192) at a concentration of 5U/µl. The microsatellites *XRCC1, XRCC3* and *XRCC5* were analysed using the QIAGEN multiplex PCR kit (QIAGEN, Ref. 206143), which comprises of a multiplex PCR mix consisting of HotStar*Taq* DNA polymerase, dNTP mix and multiplex PCR buffer containing 6mM $MgCl_2$, pH8.7 at 20°C. Details of the mastermix prepared for the multiplex microsatellite assay are provided in Table 4.5. Following preparation, mastermixes were vortexed to mix

the contents, centrifuged at 13,000 rpm for 10 seconds to collect the mix at the bottom of the tube and then 24µl aliquoted into each PCR tube (12µl for the multiplex microsatellite assay) and 1µl of template DNA added (0.5µl for the multiplex microsatellite assay), ensuring that the DNA was pipetted directly into the mix to avoid loss. Tubes were then sealed and placed onto the PCR machine and the appropriate pre-programmed thermocycle program for the SNP under investigation commenced. PCR program details for each SNP analysed and the expected product sizes are provided in Table 4.6. At the end of the PCR run tubes were stored in the refrigerator at 4ºC.

Restriction endonuclease digestion of PCR products

Following PCR amplification of the trio genomic DNA samples with SNP specific primers, restriction endonuclease digestion was undertaken in order to observe the restriction fragment length polymorphisms present. Samples for analysis of the *XRCC1*, *XRCC3* and *XRCC5* microsatellites were not digested. The appropriate number of tubes were labelled and a SNP specific digest mastermix prepared in 1.5ml eppendorfs, with the volume required determined by multiplying the amount for one tube by the number of reaction tubes and adding 10% to each total to ensure an adequate supply. Restriction endonucleases were removed from -20°C storage for as short a time as possible to prevent loss of activity and were kept on ice. Typically, 15µl digest reactions were set up in 500µl eppendorf tubes (Starlabs, Ref. 31605-0099) which were centrifuged briefly at 13,000rpm and incubated at the recommended temperature (37ºC or 65ºC) for either 2 hours or overnight. On completion of the digestion, reactions were stored at 4^{o}C. Components of the digest mastermixes, the amount of PCR product required and the conditions for digestion are detailed for all polymorphisms studied in Table 4.7. All restriction

endonucleases utilised were obtained from New England Biolabs Inc. and are represented in Table 4.7 with their corresponding catalogue number.

Table 4.2: PCR primer sequences and corresponding melting temperatures.

Primer Name	Reference	Sequence	T_m (ºC)[1]
ADPRTT2444C Forward	Cottet *et al*., (2000)	5' CAC CAT GAT ACC TAA GTC GG 3'	57.3
ADPRT2444C Reverse	Cottet *et al*., (2000)	5' ACC CTG TTA CCT TAA TGT CAT TTT 3'	55.9
APEXT649G Forward	Hu *et al*., (2001)	5' CTG TTT CAT TTC TAT AGG CTA 3'	57.6
APEXT649G Reverse	Hu *et al*., (2001)	5' AGG AAC TTG CGA AAG GCT TC 3'	54.3
HOGG1C1285G Forward	Wikman *et al*., (2000)	5' ACT GTC ACT AGT CTC ACC AG 3'	57.3
HOGG1C1285G Reverse	Wikman *et al*., (2000)	5' GGA AGG TGC TTG GGG AAT 3'	56.0
HOGG1G445A Forward	Wikman *et al*., (2000)	5' GTC TTT GGG CGT CGA CGA GGC T 3'	65.8
HOGG1G445A Reverse	Wikman *et al*., (2000)	5' ACA GGC TTC TCA GGC TCA GT 3'	59.4
XPDG935A Forward	Lunn *et al*., (2000)	5' CTG TTG GTG GGT GCC CGT ATC TGT TGG TCT 3'	70.9
XPDG935A Reverse	Lunn *et al*., (2000)	5' TAA TAT CGG GGC TCA CCC TGC AGC ACT TCC T 3'	70.8
XPDA2251C Forward	Spitz *et al*., (2001)	5' GCC CGC TCT GGA TTA TAC G 3'	58.8
XPDA2251C Reverse	Spitz *et al*., (2001)	5' CTA TCA TCT CCT GGC CCC C 3'	61.0
XRCC1G1301A Forward	Lunn *et al*., (1999)	5' TTG TGC TTT CTC TGT GTC CA 3'	56.9
XRCC1G1301A Reverse	Lunn *et al*., (1999)	5' TCC TCC AGC CTT TTC TGA TA 3'	54.1
XRCC1C685T Forward	Lunn *et al*., (1999)	5' GCC CCG TCC CAG GTA 3'	56.0
XRCC1C685T Reverse	Lunn *et al*., (1999)	5' AGC CCC AAG ACC CTT TCA CT 3'	59.4

XRCC1G944A Forward	Lunn *et al*., (1999)	5’ TTG ACC CCC AGT GGT GCT AA 3’	63.5
XRCC1G944A Reverse	Lunn *et al*., (1999)	5’ CGC TGG GAC CAC CTG TGT T 3’	64.6
XRCC1 microsatellite Forward	Price *et al*., (1997)	5’ FAM - CCC GAT GGA TCT ACA GTT GC 3’	63.6
XRCC1 microsatellite Reverse	Price *et al*., (1997)	5’ CCC AGG GAG CCT CTT AGA GT 3’	61.4
XRCC2C139T Forward*	Not applicable	CCTTGACCAACTGCCAAAACCCTTT[C/T}CAAACCCCAAGTCTATGAACCGGAC	N/A
XRCC2C139T Reverse*	Not applicable	[VIC/FAM]	N/A
XRCC3C1075T Forward	Shen *et al*., (1998)	5’ GGT CGA GTG ACA GTC CAA AC 3’	59.4
XRCC3C1075T Reverse	Shen *et al*., (1998)	5’ CTA CCC GCA GGA GCC GGA GG 3’	61.4
XRCC3 microsatellite Forward	Price *et al*., (1997)	5’ GAC AAT ATG CAT GTA TTA CTT TG 3’	54.8
XRCC3 microsatellite Reverse	Price *et al*., (1997)	5’ TET - GTG TGC AGT TTA TAT AAG GCA GG 3’	56.1
XRCC4T401C Forward	Ford *et al*., (2000)	5’ TET - CTC AGA AGA AAT TGT GTA TGC T 3’	59.6
XRCC4T401C Reverse	Ford *et al*., (2000)	5’ ACC ACA AGC AAA CTG TGT ACA C 3’	58.7
XRCC4G921T Forward	Ford *et al*., (2000)	5’ TCT CTA AAC CAA TTT GAA ACA GGA 3’	55.9
XRCC4G921T Reverse	Ford *et al*., (2000)	5’ CAG ACA GGA TGT TGG ACA GC 3’	59.4
XRCC5 microsatellite Forward	Price *et al*., (1997)	5’ TGT TGC TAT TGT TGT CTA GC 3’	54.1
XRCC5 microsatellite Reverse	Price *et al*., (1997)	5’ TET - AAG TCA CTC ACA TGT AAT CC 3’	57.6

[1] Melting temperature of primer sequence, * See text.

Table 4.3: Recipe for XRCC1/XRCC3/XRCC5 microsatellite assay 10x primer mix.

Primer (50pmol/µl)	Volume (µl)
XRCC1 microsatellite Forward	20.0
XRCC1 microsatellite Reverse	20.0
XRCC3 microsatellite Forward	20.0
XRCC3 microsatellite Reverse	20.0
XRCC5 microsatellite Forward	20.0
XRCC5 microsatellite Reverse	20.0
1x TE buffer	380.0
Total volume	500.0

Table 4.4: Recipes for PCR mastermixes (volumes required in µl for a single PCR reaction tube).

Polymorphism	dH_20	Buffer (Type)	2mM dNTPs	F primer	R primer	Formamide	$MgCl_2$	*Taq*	Final volume
ADPRT t2444c	17.84	2.50 (I)	2.50	0.50	0.50	-	-	0.16	24.00
APEX t649g	16.34	2.50 (IV)	2.50	0.50	0.50	-	1.50	0.16	24.00
hOGG1 c1285g	16.60	2.50 (I)	2.50	0.50	0.50	1.24	-	0.16	24.00
hOGG1 g445a	16.60	2.50 (V)	2.50	0.50	0.50	1.24	-	0.16	24.00
XPD g935a	16.60	2.50 (I)	2.50	0.50	0.50	1.24	-	0.16	24.00
XPD a2251c	16.60	2.50 (I)	2.50	0.50	0.50	1.24	-	0.16	24.00
XRCC1 g1301a	16.60	2.50 (V)	2.50	0.50	0.50	1.24	-	0.16	24.00
XRCC1 c685t	16.60	2.50 (V)	2.50	0.50	0.50	1.24	-	0.16	24.00
XRCC1 g944a	17.84	2.50 (I)	2.50	0.50	0.50	-	-	0.16	24.00
XRCC3 c1075t	16.60	2.50 (V)	2.50	0.50	0.50	1.24	-	0.16	24.00
XRCC4 t401c	17.34	2.50 (V)	2.50	0.50	0.50	0.50	-	0.16	24.00
XRCC4 g921t	16.60	2.50 (I)	2.50	0.50	0.50	1.24	-	0.16	24.00

Table 4.5: Recipe for XRCC1/XRCC3/XRCC5 microsatellite assay PCR mastermix.

2x mastermix	6.25µl
10x primer mix	1.25µl
dH_2O	4.50µl
Total volume	12.00µl

Table 4.6: PCR conditions for SNP analysis.

Polymorphism	PCR conditions							
	Initial activation	Denaturation	Primer annealing	DNA extension	Cycle number	Final extension	Storage	Product size
ADPRT t2444c	94ºC - 5 mins	94ºC – 30 secs	52ºC – 30 secs	72ºC – 30 secs	30	72ºC – 5 mins	4ºC - ∞ hold	181 bp
APEX t649g	95ºC - 2 mins	94ºC – 15 secs	57ºC – 45 secs	72ºC – 45 secs	40	72ºC – 5 mins	4ºC - ∞ hold	166 bp
hOGG1 c1285g	94ºC - 5 mins	94ºC – 30 secs	55ºC – 90 secs	72ºC – 60 secs	33	72ºC – 7 mins	4ºC - ∞ hold	200 bp
hOGG1 g445a	94ºC - 5 mins	94ºC – 30 secs	57ºC – 90 secs	72ºC – 60 secs	33	72ºC – 7 mins	4ºC - ∞ hold	232 bp
XPD g935a	94ºC - 5 mins	94ºC – 45 secs	60ºC – 45 secs	72ºC – 60 secs	40	72ºC – 7 mins	4ºC - ∞ hold	757 bp
XPD a2251c	94ºC - 5 mins	94ºC – 45 secs	60ºC – 45 secs	72ºC – 60 secs	30	72ºC – 7 mins	4ºC - ∞ hold	436 bp
XRCC1 g1301a	94ºC - 5 mins	94ºC – 30 secs	58ºC – 90 secs	72ºC – 30 secs	33	72ºC – 7 mins	4ºC - ∞ hold	615 bp
XRCC1 c685t	94ºC - 5 mins	94ºC – 15 secs	57ºC – 45 secs	72ºC – 45 secs	40	72ºC – 5 mins	4ºC - ∞ hold	491 bp
XRCC1 g944a	94ºC - 4 mins	94ºC – 30 secs	58ºC – 30 secs	72ºC – 30 secs	30	72ºC – 5 mins	4ºC - ∞ hold	861 bp
XRCC3 c1075t	94ºC - 5 mins	94ºC – 30 secs	52ºC – 45 secs	72ºC – 120 secs	35	72ºC – 3 mins	4ºC - ∞ hold	415 bp
XRCC4 t401c	94ºC - 5 mins	94ºC – 30 secs	52ºC – 30 secs	72ºC – 30 secs	40	72ºC – 7 mins	4ºC - ∞ hold	276 bp
XRCC4 g921t	94ºC - 5 mins	94ºC – 30 secs	57ºC – 30 secs	72ºC – 30 secs	35	72ºC – 7 mins	4ºC - ∞ hold	446 bp
XRCC1/3/5	95ºC – 15 mins	94ºC – 30 secs	57ºC – 90 secs	72ºC – 60 secs	25	60ºC – 30 mins	4ºC - ∞ hold	181, 204, 350 bp

Table 4.7: Recipes for restriction endonuclease digestion mastermixes (volumes required for a single reaction in µl).

Polymorphism	dH_20	NEB Buffer (Type)	Enzyme (Cat No.)	Volume	BSA	PCR product	Digest volume	Digest conditions
ADPRT t2444c	8.00	1.50 (#3)	*Aci* I (#R0551L)	0.50	-	5.00	15.00	37ºC – 2 hours
APEX t649g	9.50	1.50 (#4)	*Bfa* I (#R0568L)	1.00	-	3.00	15.00	37ºC – overnight
hOGG1 c1285g	8.00	1.50 (#4)	*Fnu*4H I (#R0178L)	0.50	-	5.00	15.00	37ºC – 2 hours
hOGG1 g445a	-	1.50 (#2)	*Msp* I (#R0106S)	1.00	-	12.50	15.00	37ºC – 2 hours
XPD g935a	7.85	1.50 (#3)	*Sty* I (#R0500S)	0.5	0.15	5.00	15.00	37ºC – 2 hours
XPD a2251c	7.85	1.50 (#3)	*Pst* I (#R0140S)	0.50	0.15	5.00	15.00	37ºC – 2 hours
XRCC1 g1301a	-	1.50 (#2)	*Msp* I (#R0106S)	1.00	-	12.50	15.00	37ºC – 2 hours
XRCC1 c685t	-	1.50 (#2)	*Msp* I (#R0106S)	1.00	-	12.50	15.00	37ºC – 2 hours
XRCC1 g944a	8.00	1.50 (#1)	*Rsa* I (#R0167L)	0.50	-	5.00	15.00	37ºC – 2 hours
XRCC3 c1075t	6.85	1.50 (#4)	*Nla* III (#R0125L)	0.50	0.15	6.00	15.00	37ºC – 2 hours
XRCC4 t401c	9.00	1.50 (#2)	*Bsr*D I (#R0574L)	0.50	1.00	3.00	15.00	65ºC – overnight
XRCC4 g921t	8.10	1.50 (#*Taq*)	*Taq* I (R#0149S)	0.25	0.15	5.00	15.00	65ºC – 2 hours

Visualising RFLPs using agarose gel electrophoresis

Following restriction endonuclease digestion of PCR products, RFLPs were visualised using agarose gel electrophoresis with the exception of *XRCC4* t401c which was analysed using the ABI Prism 310 platform.

Small gel tanks (10 x 10cm tank size, 8 x 10cm gel size) of 50ml gel volume were used, with two combs each having 16 wells inserted into each gel. Table 4.8 provides details of the agarose gel percentage required to achieve separation of the DNA fragments and the size of the expected digest products for each polymorphism analysed. Gels were prepared by weighing the appropriate amount of agarose gel (Sigma, Ref. A9539) into a 200ml conical flask, adding 50ml of 2x TAE buffer and dissolving by microwaving at 350 watts in 30 second bursts, followed by swirling to mix, until no grains were visible in the solution. The agarose solution was allowed to cool to approximately 60ºC, ethidium bromide added at a final concentration of 0.5µg/ml and then poured slowly and carefully, avoiding the introduction of air bubbles, into the assembled gel tank to mould and the combs put into place. Once set, casting plates and combs were removed and the gel submersed in 2x TAE buffer. A 50x TAE stock solution was diluted 1:25 to produce the 2x TAE working solution required. 2x concentration was used as it provides increased buffering capacity in the small volume gel tanks. Stock solution was prepared by mixing 121g Tris (Sigma, Ref. T-6066), 28.55ml glacial acetic acid (VWR Int., Ref. 10001CU) and 50ml 0.5M EDTA pH8.0, and was made up to 500ml with distilled water and then autoclaved. Stock solution was stored at room temperature. Ethidium bromide (Sigma, Ref. E2515) at a concentration of 5mg/ml was added to the 2x TAE working solution to produce a final concentration of 0.5µg/ml.

A ‘gel plan’ detailing the digestion reactions to be loaded onto gels was prepared for reference for each gel in advance to any samples being loaded (refer to Appendix D). DNA size standards were prepared by mixing 4µl 100bp PCR ladder (Sigma, Ref. P1473) with 1µl 6x loading dye and 5µl loaded into the first lane of each agarose gel. Digestion reactions (15µl) were mixed with 3µl 6x loading dye and 10µl of each sample loaded. Samples were electrophoresed at approximately 50V for 60 minutes or longer depending on the extent of fragment separation required.

Images of gels were captured by illuminating with UV light on a digital gel documentation system (Syngene-GeneGenius Bio Imaging System) using the analysis software GeneSnap and GeneTools, and saved onto the computer with the appropriate labelling. Gels were then discarded into a clinical waste bin and any solutions containing ethidium bromide collected in an aspirator for decontamination with destaining bags (Merck, Ref. 42992 2L).

Table 4.8: Expected size of PCR digest products and percentage agarose gel used for each SNP analysed.

Polymorphism	Agarose gel %	Wild type allele	Size (bp)	Mutant allele	Size (bp)
ADPRT t2444c	3	T	181	C	129, 52
APEX t649g	4	T	166	G	144, 20
hOGG1 c1285g	3	C	200	G	100, 100
hOGG1 g445a	4	G	204, 28	A	232
XPD g935a	5	G	357, 176, 151, 73	A	357, 176, 117, 73, 34
XPD a2251c	3	A	290, 146	C	227, 146, 63
XRCC1 g1301a	3	G	374, 221	A	615
XRCC1 c685t	3	C	293, 178, 20	T	313, 178
XRCC1 g944a	2	G	580, 221, 60	A	640, 221
XRCC3 c1075t	4	C	274, 141	T	169, 141, 105
XRCC4 t401c	ABI Prism 310	T	174, 102	C	276
XRCC4 g921t	2	G	446	T	300

Genotyping the XRCC1/XRCC3/XRCC5 microsatellites and XRCC4 t401c

Genotyping the trio samples for the *XRCC1*, *XRCC3* and *XRCC5* microsatellites and the *XRCC4* t401c SNP was performed using the ABI Prism 310 genetic analyser which operates by sizing DNA fragments and determining their size by comparing them to fragments contained in a size standard between 35 and 500 bp and known as Genescan 500 TAMRA (Applied Biosystems, Ref. 401733).

Each DNA sample to be analysed was labelled with a fluorescent dye incorporated during PCR performed with a fluorescently tagged primer. The internal size standard was also pre-labelled with a fluorescent dye and since the dyes come in sets of four colours, it was possible to electrophorese three samples and a size standard simultaneously.

Initially, 0.5ml MicroAmp reaction tubes (Applied Biosystems, Ref. 401957) were labelled with the sample identification number. Size standards were then prepared by mixing 50µl TAMRA-500 with 600µl formamide and 12µl aliquoted into the bottom of each sample tube. For analysis of the *XRCC1/3/5* microsatellites, 1µl of PCR product was aliquoted into the bottom of the reaction tubes and for the *XRCC4* t401c SNP 1µl of digest diluted 1:5 with distilled water was aliquoted. Non-template controls using distilled water were also included in each run. Samples were denatured at 95°C for 3 minutes and loaded onto a MicroAmp tray and into the ABI Prism 310 instruments autosampler.

A sample sheet detailing sample numbers, the fluorophore label used on the PCR primer (FAM – blue, TET – green, HEX – yellow) and the TAMRA labelled size standard (red) was prepared using the ABI Prism 310 Collection software and saved onto the computer.

An injection list was then prepared by importing the appropriate sample sheet and adjusting the run time accordingly (24 minutes for *XRCC4* t401c and 27 minutes for *XRCC1/3/5* microsatellites). Following this the run was commenced.

Each sample was successively brought into contact with a cathode electrode, at one end of a capillary filled with a polymer for electrophoresing DNA under denaturing conditions (Performance Optimised Polymer 4, supplied by Applied Biosystems, Ref. 402838). An anode electrode at the other end of the capillary was immersed in buffer (10x Genetic Analyser Buffer with EDTA supplied by Applied Biosystems, Ref. 402824). A portion of the sample entered the capillary due to the current flowing from the cathode to the anode. The end of the capillary near the cathode was then placed in buffer and current applied again to commence electrophoresis. When the DNA fragments reached the detector window in the capillary coating, a laser excited the fluorescent dye labels. Emitted fluorescence from the dyes was collected by a CCD camera and on completion of a run, the instruments software interpreted the results and calculated the size of the fragments by comparison of the fluorescence intensity at each data point to the known TAMRA size standards.

Analysis of the trio cohort for the XRCC2 c139t SNP

In addition to previously characterised SNPs, it was decided to analyse the trio cohort for SNPs in candidate DNA repair genes that were unidentified in the literature. This involved performing searches of the NCBI (National Centre for Biotechnology Information) SNP database (**http://www.ncbi.nlm.nih.gov/**). Since the trio cohort was made up of only 100 samples, SNPs with moderately high allele frequencies were required in order to observe both mutant and wild type genotypes. From a number of potential candidates, it was decided to genotype for a SNP in the *XRCC2* gene known as *XRCC2 c139t* because polymorphisms in this gene had not been previously investigated in the institute and also because *XRCC2* is involved homologous recombination repair of double strand breaks. Table 4.1 provides details of the SNP.

In contrast to analysis by PCR-RFLP using agarose gel electrophoresis or the ABI Prism 310 platform, the trio cohort was analysed for the *XRCC2* c139t SNP using a Taqman Validated SNP Genotyping Assay which utilises a single-tube PCR assay and a post-assay allelic discrimination plate read. Applied Biosystems design such assays for use with their ABI Prism 7000 sequence detection system (SDS) instrumentation and have a vast resource of over 160,000 validated assays (De la Vega *et al.*, 2005). The advantage of validated SNP assays is that information on allele frequencies has already been determined in reference samples from major populations. This helps to alleviate time and expense problems often associated with the development of such assays due to high failure rates when selecting SNPs at random. In order to analyse the trio cohort for *XRCC2* c139t, the NCBI SNP database public identification accession number attributed to the SNP (rs1871892) was used to search the Applied Biosystems SNP Genotyping Assay database and an inventoried and validated assay was identified (Assay ID C-1141440010).

Overview of the Taqman SNP genotyping assay

The Taqman SNP genotyping assay includes two PCR primers that flank the SNP of interest and two TaqMan oligonucleotide probes for detecting alleles (refer to Figure 4.2A). These probes have a fluorescent reporter dye at the 5' end (VIC dye is linked to the 5’ end of the allele X probe and FAM to the 5’ end of the allele Y probe). They also have a non-fluorescent quencher (NFQ) and a minor groove binder (MGB) at the 3' end. The MGB increases the melting temperature (T_m) without increasing probe length and thus allows the design of shorter probes resulting in greater differences in T_m values between matched and mismatched probes, which produces more accurate allelic discrimination. The NFQ does not fluoresce and thus sequence detection systems can measure reported dye contributions more accurately.

During PCR, each TaqMan MGB probe anneals specifically to a complementary sequence between forward and reverse primer sites. An intact probe emits minimal fluorescent signal when excited, because the close physical proximity of the 5' fluorophore to the 3' quencher causes the fluorescent resonance energy transfer (FRET) effect to quench the fluorescence emitted by the fluorophore. A fluorescent signal is only generated when an intact probe, which is hybridised to the target allele, is cleaved by the 5' exonuclease activity of AmpliTaq Gold DNA Polymerase. In each PCR cycle, cleavage of one or both allele-specific probes produces an exponentially increasing fluorescent signal by freeing the 5' fluorophore from the 3' quencher and since this only occurs if the amplified target sequence is complimentary to the probe, the fluorescence signal generated thus indicates which alleles are present in a sample. The use of two probes, one specific to each allele of the SNP and labeled with two fluorophores, allows detection of both alleles in a single tube (refer to Figure 4.2B).

The TaqMan SNP Genotyping Assay is read at the PCR endpoint rather than in real time. DNA samples are genotyped simultaneously on 96-well plates and results then visualised by plotting the normalised intensity of the reporter dyes in each sample well on a scatter plot.

PCR amplification and post allelic discrimination plate read

The first step in performing the SNP genotyping assay was to prepare reaction plate plans for the MicroAmp optical 96-well reaction plates (Applied Biosystems, Ref. PN N801-0560) used. The initial plate prepared contained non-template controls (NTCs) and genomic DNA samples at 5ng, 10ng and 20ng in order to optimise reaction conditions for the subsequent samples. The recommended template for TaqMan SNP Genotyping Assays is purified genomic DNA at 1 to 20ng and following the run of the first plate, it was decided to use 20ng template DNA. In order to assay the entire trio cohort plus 10% repeat samples for quality control purposes it was necessary to set up a further two reaction plates.

Initially, the reaction mix for PCR amplification was prepared. The reaction mix contained 2x TaqMan Universal PCR Master Mix consisting of AmpliTaq Gold DNA Polymerase, dNTPs and optimised buffer components (Applied Biosystems, Ref. PN 4324018) and 20x TaqMan SNP Genotyping Assay Mix (Applied Biosystems, Ref. PN 4331183). TaqMan Universal PCR Master Mix was removed from the refrigerator and thoroughly mixed by swirling the bottle. Assay mix was removed from the -20ºC freezer and allowed to thaw before being resuspended by vortexing and centrifuged briefly to spin down the contents. Assay mix was wrapped in tin foil to protect the fluorescent probes from the effects of light exposure. For 96-well plates, a 25µl volume reaction per well is

recommended. The number of reactions to be performed for each assay and the required quantity of reaction mix plus 10% to provide excess volume for any losses during reagent transfer were determined. The mix was prepared by pipetting the required volumes of each reagent into a sterile tube, inverting to mix and centrifuging briefly to spin down the contents and eliminate any air bubbles from the solution. DNA concentrations of the trio samples were quantified using a biophotometer and where necessary diluted to 20ng with DNase-free water. Reaction mix for PCR was aliquoted into the bottom of each well on a reaction plate and genomic DNA delivered to the final mix (Table 4.9). Pipetting accuracy was checked by observing each well and noting which did not appear to contain the correct volume. Finally, reaction plates were covered with an optical adhesive cover (Applied Biosystems, Ref. PN 4313663) before being loaded into the ABI Prism 7000 SDS and the appropriate thermal cycling conditions for PCR amplification selected (Table 4.10).

After PCR amplification, a post-assay allelic discrimination endpoint plate read was performed by creating a plate read document in which detectors (alleles X and Y) were assigned reporter dyes (VIC and FAM) and a colour (yellow and red) for ease of identification. SDS software was then used to calculate the fluorescence measurements made and plot the values based on signals from each well. The data was plotted as crosses representing fluorescence for the VIC-labelled probe on the x-axis (allele X) plotted against fluorescence for the FAM-labelled probe on the y-axis (allele Y) and alleles then called depending on their respective fluorescence. In the case of *XRCC2* c139t, allele X represented the C nucleotide, whilst allele Y represented the T nucleotide.

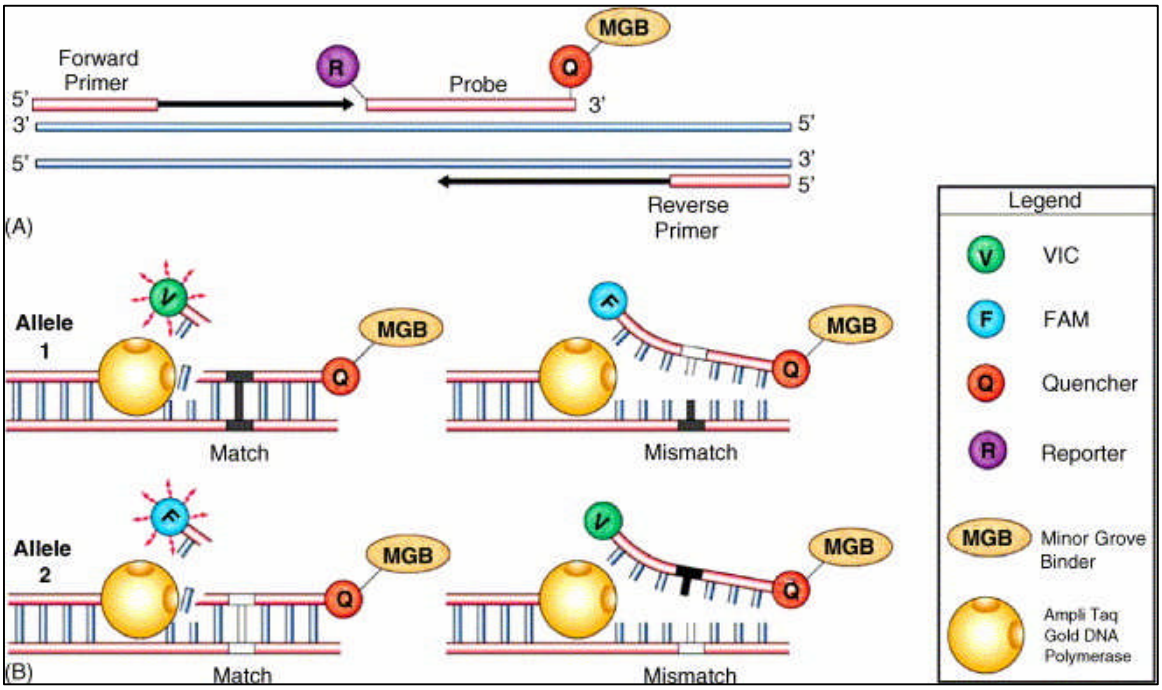


Figure 4.2: (A) Probe binding and primer extension in a Taqman SNP Genotyping Assay. (B) Allelic discrimination is achieved by the selective annealing of matching probe and template sequences, which generates an allele specific fluorescent signal. Figure adapted from De La Vega et al. (2005).

Table 4.9: Components required for PCR assay.

Component	Volume (µl)
2x TaqMan Universal PCR Master Mix	12.50
20x TaqMan SNP Genotyping Assay Mix	1.25
20ng genomic DNA (diluted in DNase-free water)	11.25
Total	25.00

Table 4.10: Thermal cycling conditions for PCR.

	AmpliTaq Gold Activation	PCR conditions	
	HOLD	CYCLE (40 CYCLES)	
		Denature	Anneal/Extend
Time	10 min	15 sec	1 min
Temp	95ºC	92ºC	60ºC

Statistical analysis

Genotype and allele frequency analyses

Since codominant inheritance was observed at all loci, allele frequencies were determined from genotype data using the gene counting method (Li, 1976). Conformity of genotype frequencies to Hardy-Weinberg equilibria were tested using the exact test method of Guo & Thompson (1992) and the web-page http://www2.biology.ualberta.ca/jbrzusto/hwenj.html. For the three microsatellite loci, a large number of possible genotypes were observed and thus, alleles were classified as either common or rare. Rare microsatellites were alleles with less than 12 or more than 23 repeats for *XRCC1*, alleles with more than 20 repeats for *XRCC3*, and alleles with less than 14 or more than 18 repeats for *XRCC5*. In these instances, allele count data were used in subsequent calculations.

Genotype data for the 13 SNPs and allele count data for the three microsatellites were analysed for frequency differences amongst three groups: survivors of childhood and adolescent cancer, their partners and offspring; survivors of childhood and adolescent cancer and their partners; and finally parents (pooled data on survivors of childhood and adolescent cancer and their partners) and their offspring. These analyses were performed by considering the genotype data in the relevant groups in the form of r x c contingency tables, for each of which the empirical levels of significance were computed by the permutation test using the algorithm described in Roff & Bentzen (1989). For each test, *P*-values were obtained from 10,000 replications of permutations.

Genotype and G_2 chromosomal radiosensitivity analyses

Association of polymorphism with G_2 chromosomal radiosensitivity was determined by two methods. Initially, analysis of variance (one-way ANOVA performed using Minitab15 software) was used to test if the mean values of G_2 chromosomal sensitivity were significantly different for the genotypes at each polymorphic site. In this instance, data from childhood and adolescent cancer survivors, their partners and offspring were pooled to form a single sample. *P*-values (indicating any possible signature of association) and R^2 values (proportion of variance explained by genotypic difference) were examined. Secondly, a more sophisticated statistical analysis was undertaken by Professor Ranajit Chakraborty and his colleagues at the Centre for Genome Information, University of Cincinnati, USA. This involved implementing a test of no-association and no-linkage using the family-based association statistic of the FBAT software (version 1.5.5), the theoretical rationale for which is described in Horvath *et al.* (2001). For both types of analysis, the offspring with a G_2 score of 406 chromatid aberrations per 100 cells was excluded. Finally, since several of the polymorphic sites are linked, the FBAT analyses were also repeated at the haplotype level for five genes (*hOGG1*, *XRCC1*, *XRCC4*, *XRCC3*, and *XPD*), for which the FBAT routine internally constructs the haplotypes based on multiple tightly linked markers. The test statistic and *P*-value were computed treating the haplotypes as multiple alleles (described in FBAT-toolkit user manual).

Results

Genotype and allele frequencies

Single nucleotide polymorphism analysis was performed on all 28 families received from Denmark. As previously described in Chapter 3, three families were excluded from the study due to failure to culture in the G_2 assay, one family was excluded through lack of offspring samples and one sample was excluded due to cancer being removed from the final diagnosis. Thus, genotype data were obtained for 23 families and are presented in Table 4.11, together with probabilities of observing each genotype using χ^2 analysis and *P*–values for testing conformity of genotype frequencies to the Hardy-Weinberg equilibrium (HWE) using the exact test method. One offspring sample (605) was excluded due having an unusually high G_2 score of 406 chromatid aberrations per 100 cells. Therefore, results are presented for a total of 37 offspring. For *XRCC2* c139t, three samples failed PCR analyses and thus genotypes for 22 partners and 35 offspring were obtained for this SNP. The exact test of HWE could not be performed on five SNPs (*hOGG1* g445a, *XRCC1* c685t, *XRCC1* g944a, *XRCC4* t401c and *XRCC4* g921t) due to a lack of mutant homozygotes in the donor groups. Analyses of genotype frequencies revealed no statistically significant deviations from HWE expectations in any of the donor groups. Allele frequencies are also given in Table 4.11. Allele counts for the microsatellite loci examined are shown in Table 4.12, with *XRCC1*, *XRCC3*, and *XRCC5* displaying 8, 9 and 8 different allele sizes, respectively.

Comparison of genotype frequencies amongst cancer survivors, their partners and offspring revealed no statistically significant differences in the 13 SNPs analysed, except for *APEX* t649g ($P = 0.008$) (refer to Table 4.13). On further examination of genotype

frequencies at this SNP (refer to Figure 4.3), a statistically significant difference was also observed between cancer survivors and their partners ($P = 0.001$), but not between parents and their offspring ($P = 0.753$). However, following correction for multiple testing (using Bonferroni adjustment for 16 tests) the observed significance ($P = 0.008$) did not remain significant at the 5% level, but cancer survivor and partner differences did ($P = 0.016$). In contrast to genotype frequencies, comparison of allele frequencies between the donor groups for the three microsatellite loci analysed, did not highlight any statistically significant differences for any of the three combinations tested.

Table 4.11: Genotype/allele frequencies for 13 SNPs investigated in childhood and adolescent cancer survivors, partners and offspring.

Polymorphism		WT/WT[1]	WT/M[1]	M/M[1]	*P* (obs)[2]	f(WT)[3]	f(M)[3]	P_{HWE}[4]
ADPRT t2444c	Genotype	T/T	T/C	C/C		f(T)	f(C)	
	Survivors (n = 23)	14	8	1	0.462	0.78	0.22	1.000
	Partners (n = 23)	14	8	1	0.462	0.78	0.22	1.000
	Offspring (n = 37)	25	10	2	0.275	0.81	0.19	0.584
APEX t649g	Genotype	T/T	T/G	G/G		f(T)	f(G)	
	Survivors (n = 23)	7	16	0	-	0.65	0.35	-
	Partners (n = 23)	2	11	10	0.357	0.33	0.67	1.000
	Offspring (n = 37)	6	20	11	0.231	0.43	0.57	0.739
hOGG1 c1285g	Genotype	C/C	C/G	G/G		f(C)	f(G)	
	Survivors (n = 23)	14	6	3	0.113	0.74	0.26	0.128
	Partners (n = 23)	18	4	1	0.287	0.87	0.13	0.310
	Offspring (n = 37)	20	16	1	0.244	0.76	0.24	0.652
hOGG1 g445a	Genotype	G/G	G/A	A/A		f(G)	f(A)	
	Survivors (n = 23)	23	0	0	-	1.00	0.00	-
	Partners (n = 23)	22	1	0	-	0.98	0.02	-
	Offspring (n = 37)	36	1	0	-	0.99	0.01	-

XRCC1 c685t	Genotype	C/C	C/T	T/T		f(C)	f(T)	
	Survivors (n = 23)	22	1	0	-	0.98	0.02	-
	Partners (n = 23)	18	5	0	-	0.89	0.11	-
	Offspring (n = 37)	32	5	0	-	0.93	0.07	-
XRCC1 g944a	Genotype	G/G	G/A	A/A		f(G)	f(A)	
	Survivors (n = 23)	22	1	0	-	0.98	0.02	-
	Partners (n = 23)	17	6	0	-	0.87	0.13	-
	Offspring (n = 37)	31	6	0	-	0.92	0.08	-
XRCC1 g1301a	Genotype	G/G	G/A	A/A		f(G)	f(A)	
	Survivors (n = 23)	10	8	5	0.134	0.61	0.39	0.208
	Partners (n = 23)	13	8	2	0.339	0.74	0.26	0.609
	Offspring (n = 37)	14	16	7	0.209	0.59	0.41	0.512
XRCC4 t401c	Genotype	T/T	T/C	C/C		f(T)	f(C)	
	Survivors (n = 23)	18	5	0	-	0.89	0.11	-
	Partners (n = 23)	22	1	0	-	0.98	0.02	-
	Offspring (n = 37)	33	4	0	-	0.95	0.05	-
XRCC4 g921t	Genotype	G/G	G/T	T/T		f(G)	f(T)	
	Survivors (n = 23)	18	5	0	-	0.89	0.11	-

	Partners (n = 23)	19	4	0	-	0.91	0.09	-
	Offspring (n = 37)	31	6	0	-	0.92	0.08	-
XRCC2 c139t	Genotype	C/C	C/T	T/T		f(C)	f(T)	
	Survivors (n = 23)	11	9	3	0.298	0.67	0.33	0.643
	Partners (n = 22)	9	9	4	0.265	0.61	0.39	0.653
	Offspring (n = 35)	15	17	3	0.271	0.67	0.33	0.712
XRCC3 c1075t	Genotype	C/C	C/T	T/T		f(C)	f(T)	
	Survivors (n = 23)	11	10	2	0.381	0.70	0.30	1.000
	Partners (n = 23)	9	12	2	0.307	0.65	0.35	0.662
	Offspring (n = 37)	19	16	2	0.301	0.73	0.27	1.000
XPD g935a	Genotype	G/G	G/A	A/A		f(G)	f(A)	
	Survivors (n = 23)	16	5	2	0.150	0.80	0.20	0.165
	Partners (n = 23)	13	7	3	0.173	0.72	0.28	0.299
	Offspring (n = 37)	21	16	0	0.130	0.78	0.22	0.164
XPD a2251c	Genotype	A/A	A/C	C/C		f(A)	f(C)	
	Survivors (n = 23)	10	11	2	0.357	0.67	0.33	1.000
	Partners (n = 23)	8	12	3	0.324	0.61	0.39	1.000
	Offspring (n = 37)	18	15	4	0.272	0.69	0.31	0.712

[1] WT = wild type, M = mutant; [2] Probability of the observed genotype using χ^2 analysis; [3] f(WT) = wild type allele frequency, f(M) = mutant allele frequency; [4] Probability of observed genotype using exact method.

Table 4.12: Allele counts for the microsatellite loci XRCC1, XRCC3 AND XRCC5 investigated in childhood and adolescent cancer survivors, their partners and offspring.

	XRCC1			*XRCC3*			*XRCC5*		
Allele Size	Survivor	Partner	Offspring	Survivor	Partner	Offspring	Survivor	Partner	Offspring
11	1	4	4						
12				2	2	1			
13				6	6	8			
14				8	9	13	2		
14+2								1	
15				11	12	17	19	20	31
15+2							3		1
16	4		3	13	12	22	17	18	33
16+2							1	1	2
17	16	14	26	2	2	5	4	5	6
18	15	16	24					1	1
19	5	6	7	1	1	2			
20	1	3	3	2	2	5			
21	3	3	7	1		1			
22	1								

Table 4.13: Comparison of genotype/allele frequencies between childhood and adolescent cancer survivors, their partners and offspring.

Polymorphism	*P* value		
	Survivors, partners, offspring	Survivors v partners	Parents v offspring
ADPRT t2444c	0.962	1.000	0.806
APEX t649g	0.008	0.001	0.753
hOGG1 c1285g	0.120	0.458	0.079
hOGG1 g445a	0.757	1.000	1.000
XPD g935a	0.142	0.685	0.051
XPD a2251c	0.879	0.838	0.705
XRCC1 g1301a	0.598	0.444	0.564
XRCC1 c685t	0.221	0.189	1.000
XRCC1 g944a	0.128	0.093	1.000
XRCC2 c139t	0.839	0.924	0.666
XRCC3 c1075t	0.915	0.904	0.745
XRCC4 t401c	0.193	0.182	1.000
XRCC4 g921t	0.939	1.000	0.780
XRCC1 microsatellite	0.754	0.328	0.996
XRCC3 microsatellite	1.000	1.000	0.966
XRCC5 microsatellite	0.441	0.460	0.889

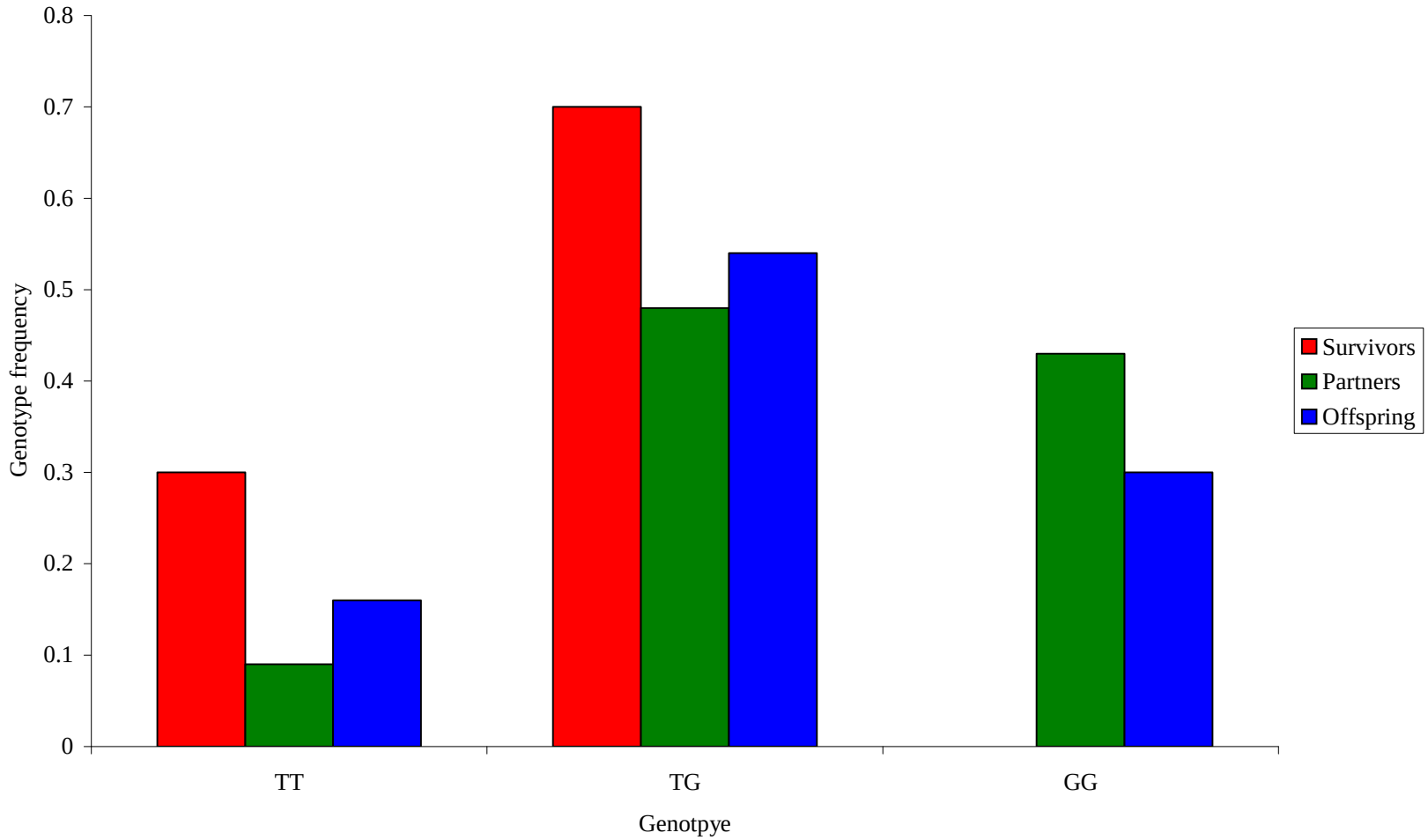


Figure 4.3: Comparison of genotype frequencies amongst cancer survivors, their partners and offspring at the APEX t649g SNP (Wild-type: TT, wild-type/mutant: TG, mutant: GG).

Examining the association between G_2 chromosomal radiosensitivity and single nucleotide polymorphisms in DNA repair genes.

Mean chromatid aberration frequencies for the Danish trios observed at the given genotypes for each of the 13 SNPs and three microsatellites analysed are illustrated in Figures 4.4 a–p. One way analysis of variance revealed no statistically significant differences in the mean values of G_2 chromatid aberrations for the given genotypes at each SNP, except for *XRCC3* c1075t (P = 0.030) (refer to Table 4.14). In this instance, individuals displaying the CC genotype had a statistically significant higher mean G_2 score of 129.74 ± 5.74, when compared to individuals with the CT and TT genotypes with mean G_2 scores of 113.47 ± 3.99 and 113.00 ± 12.82, respectively. Variance of the G_2 score at the *XRCC3* c1075t SNP site was 8.42%. Albeit this, the result did not remain significant at the 5% level following Bonferroni adjustment for multiple testing.

FBAT analysis of the data revealed statistically significant departure from the no linkage and no association hypothesis at the *hOGG1* c1285g SNP only (χ^2_2 = 4.254, P = 0.039), although this did not remain significant at the 5% level following multiple test adjustment (for 16 tests) using the Bonferroni method (refer to Table 4.14). Haplotype-based analyses (HBAT) were also undertaken in order to determine whether sets of SNPs on a single chromatid were statistically associated with G_2 radiosensitivity scores. Table 4.15 shows the summary of results for HBAT analyses for all five genes where more than one SNP was genotyped in the Danish trio donors. A statistical association was not found between any of the SNPs in these five genes at the 5% level, although the *hOGG1* result was borderline (χ^2 = 5.254, with P = 0.072).

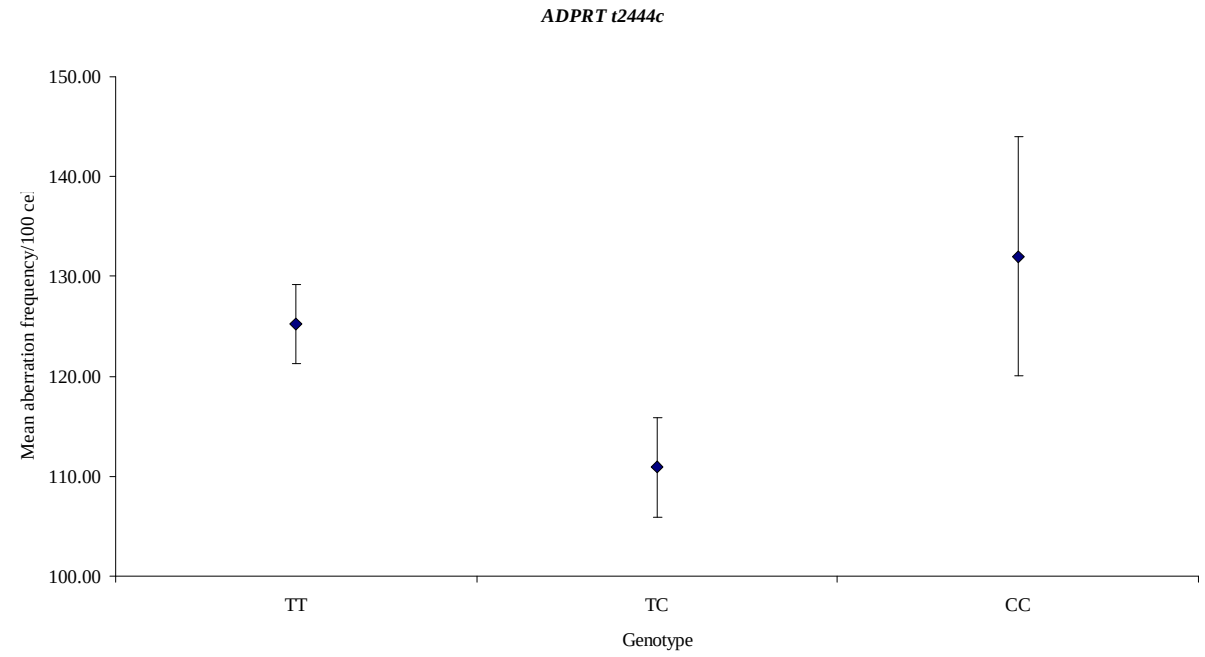


Figure 4.4 a): Association of G_2 radiosensitivity with genotype for ADPRT t2444c.

Wild-type: T / T, wild-type / mutant: T / C, mutant: C / C.

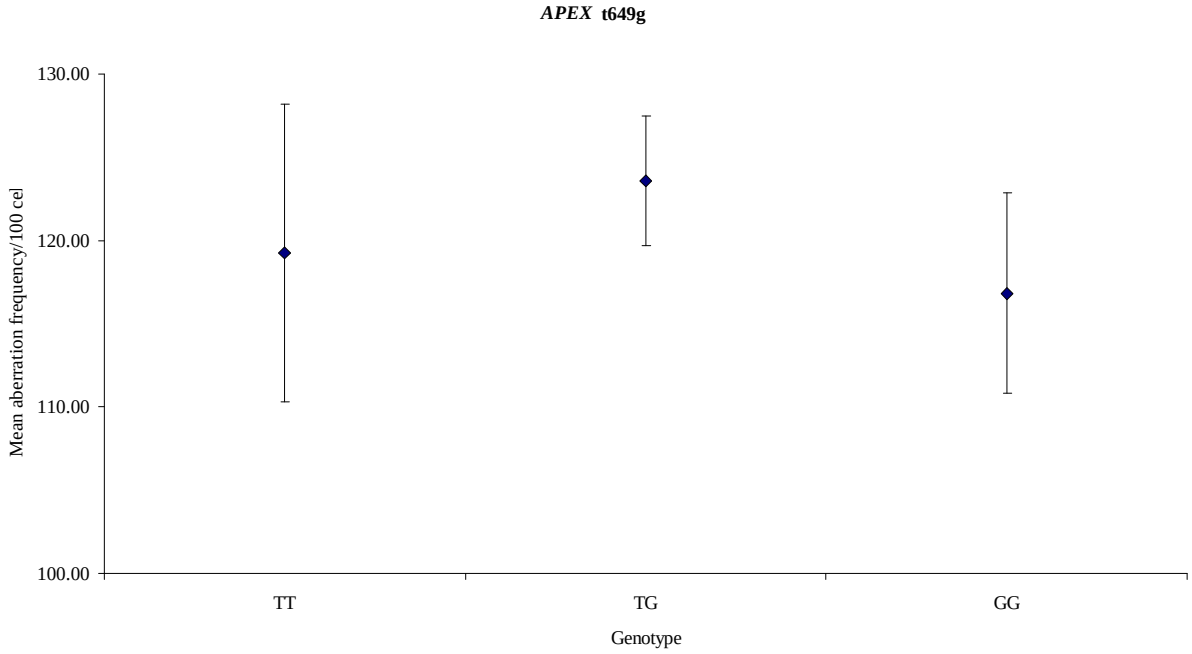


Figure 4.4 b): Association of G_2 radiosensitivity with genotype for APEX t649g.

Wild-type: T / T, wild-type / mutant: T / G, mutant: G / G.

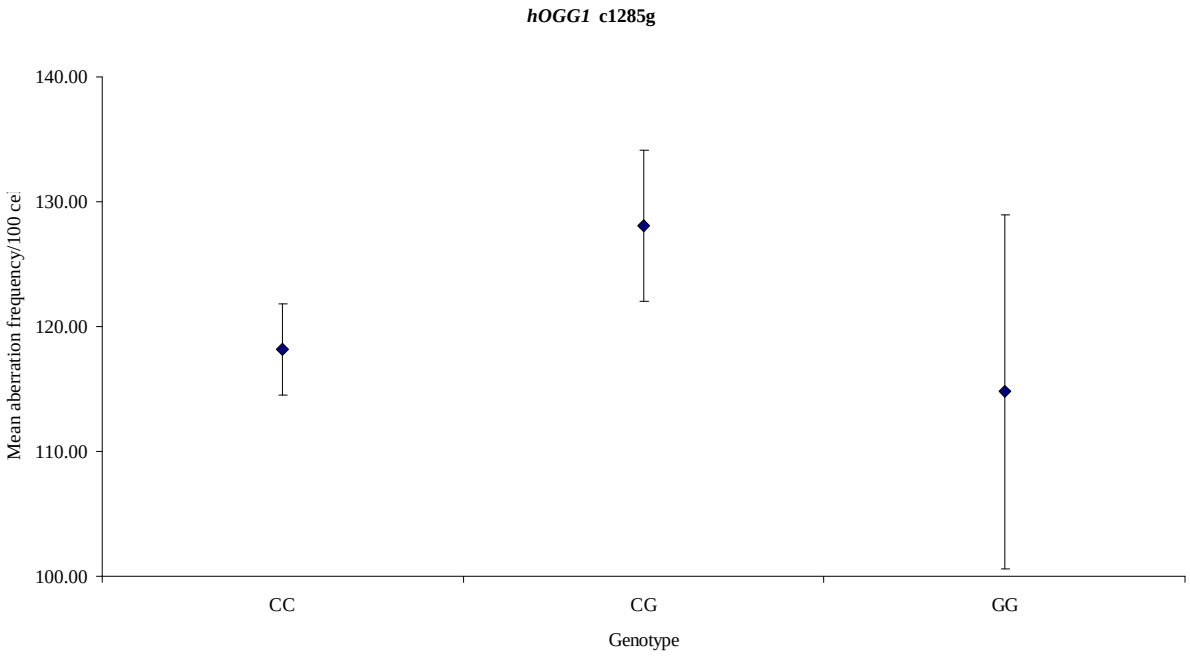


Figure 4.4 c): Association of G_2 radiosensitivity with genotype for hOGG1 c1285g.

Wild-type: C / C, wild-type / mutant: C / G, mutant: G / G.

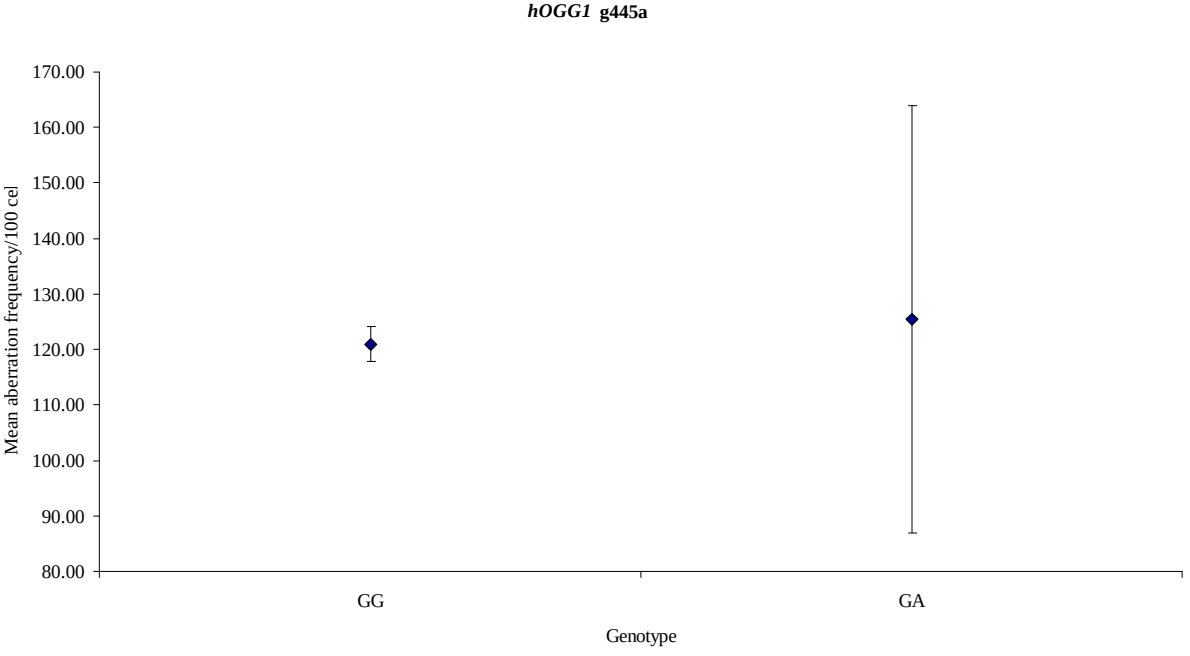


Figure 4.4 d): Association of G_2 radiosensitivity with genotype for hOGG1 g445a.

Wild-type: G / G, wild-type / mutant: G / A, mutant: A / A (non observed).

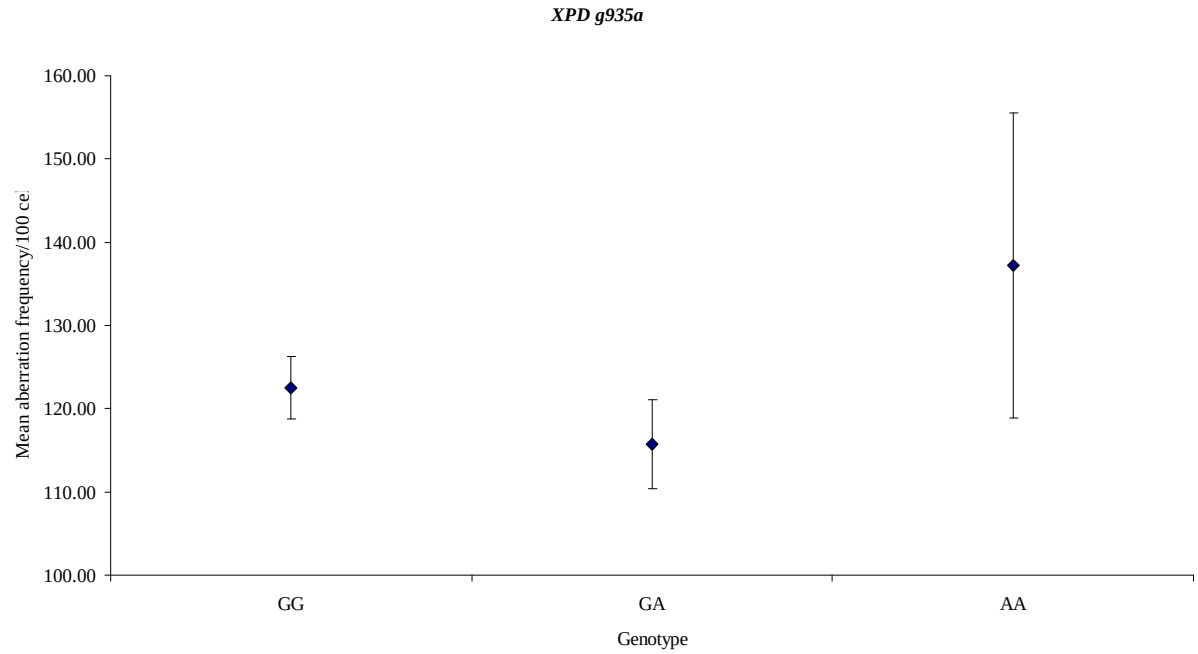


Figure 4.4 e): Association of G_2 radiosensitivity with genotype for XPD g935a.

Wild-type: G / G, wild-type / mutant: G / A, mutant: A / A

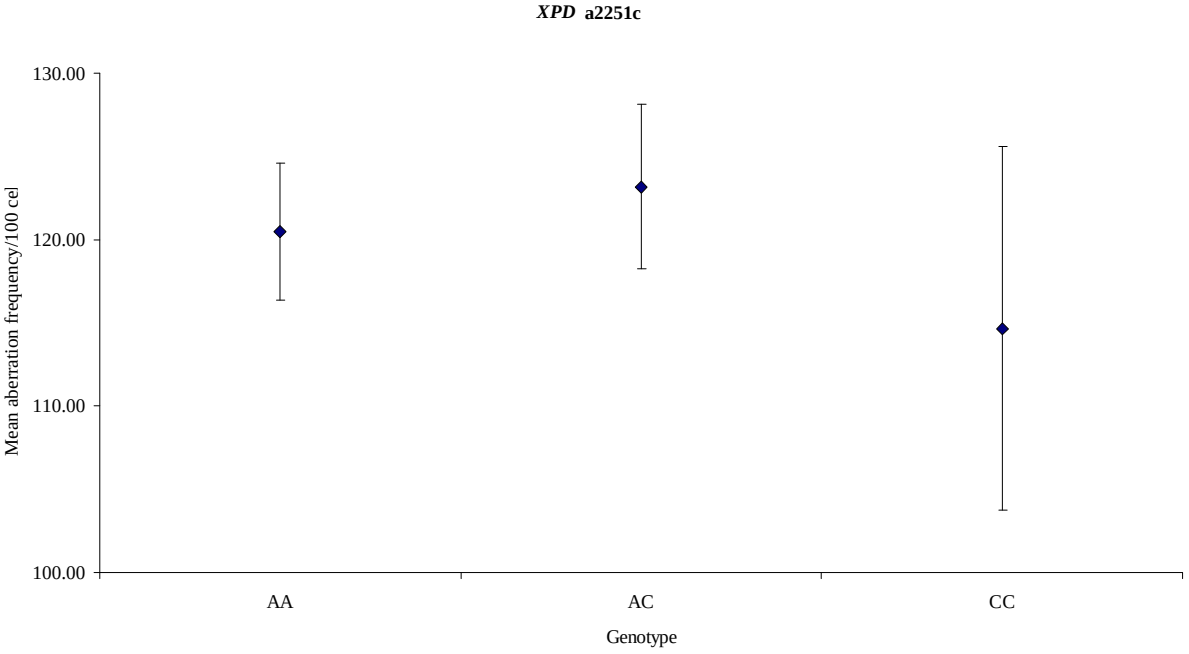


Figure 4.4 f): Association of G_2 radiosensitivity with genotype for XPD a2251c.

Wild-type: A / A, wild-type / mutant: A / C, mutant: C / C.

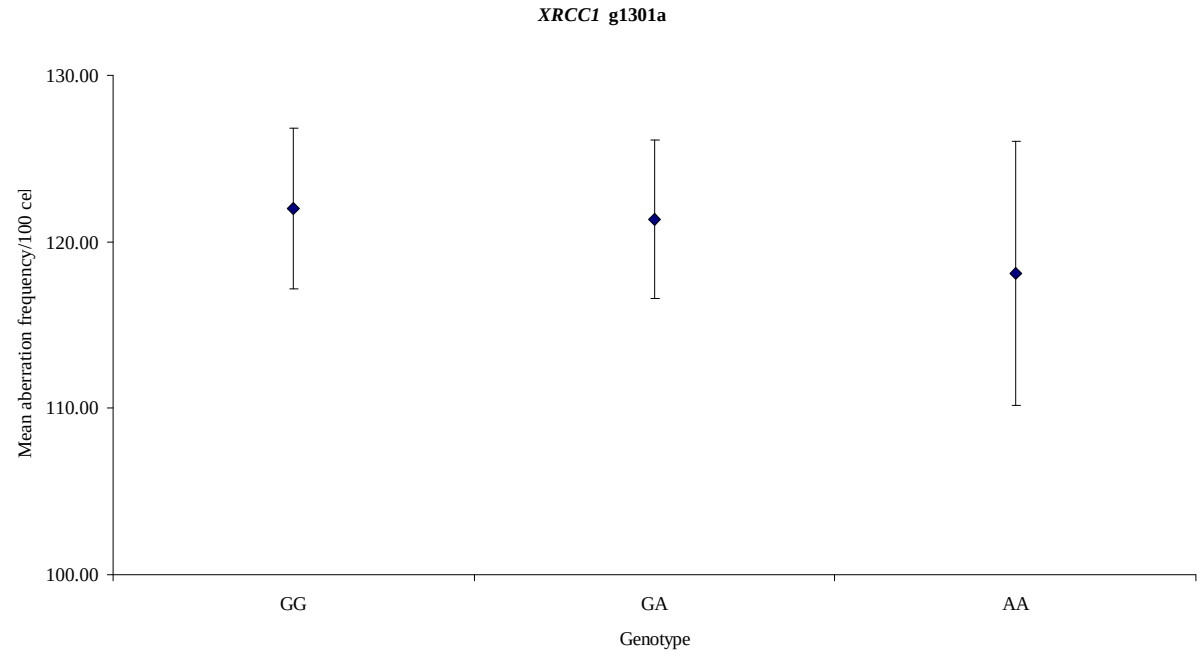


Figure 4.4 g): Association of G_2 radiosensitivity with genotype for XRCC1 g1301a.

Wild-type: G / G, wild-type / mutant: G / A, mutant: A / A.

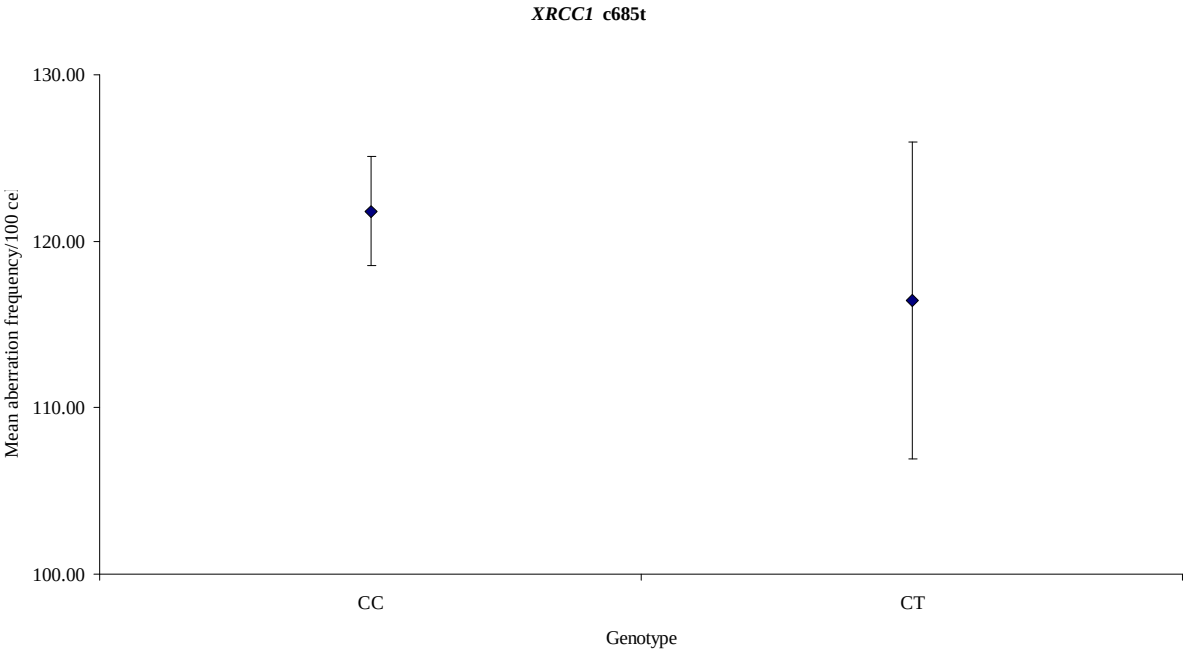


Figure 4.4 h): Association of G_2 radiosensitivity with genotype for XRCC1 c685t.

Wild-type: C / C, wild-type / mutant: C / T, mutant: T / T (non observed).

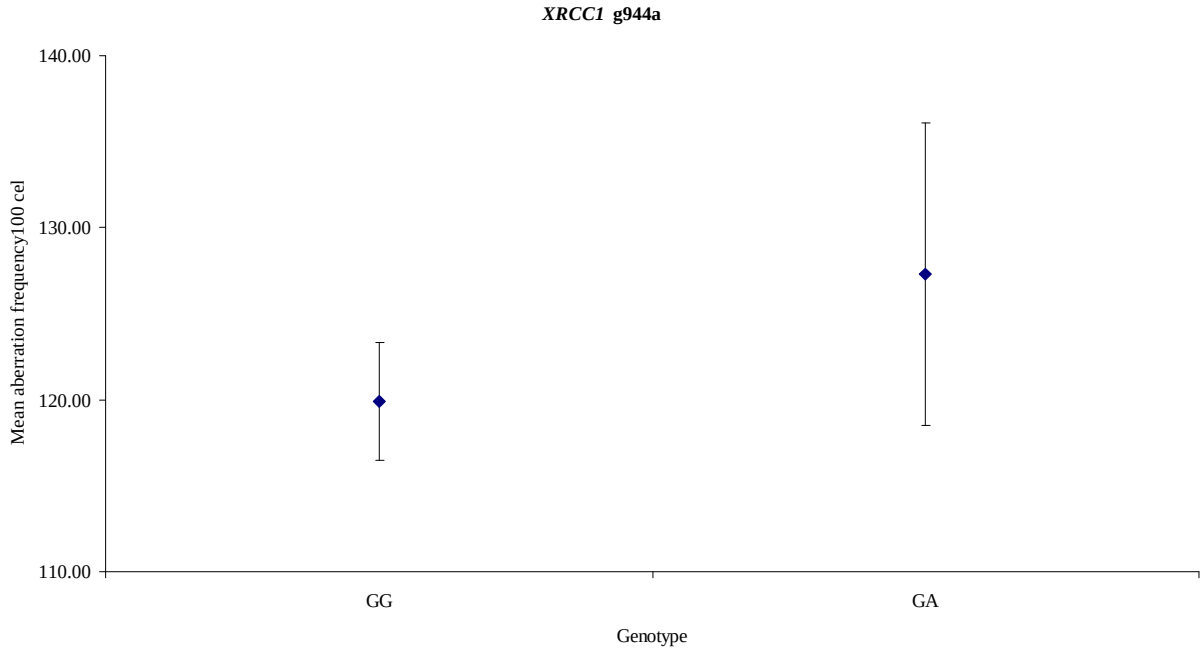


Figure 4.4 i): Association of G_2 radiosensitivity with genotype for XRCC1 g944a.

Wild-type: G / G, wild-type / mutant: G / A, mutant: A / A (non observed).

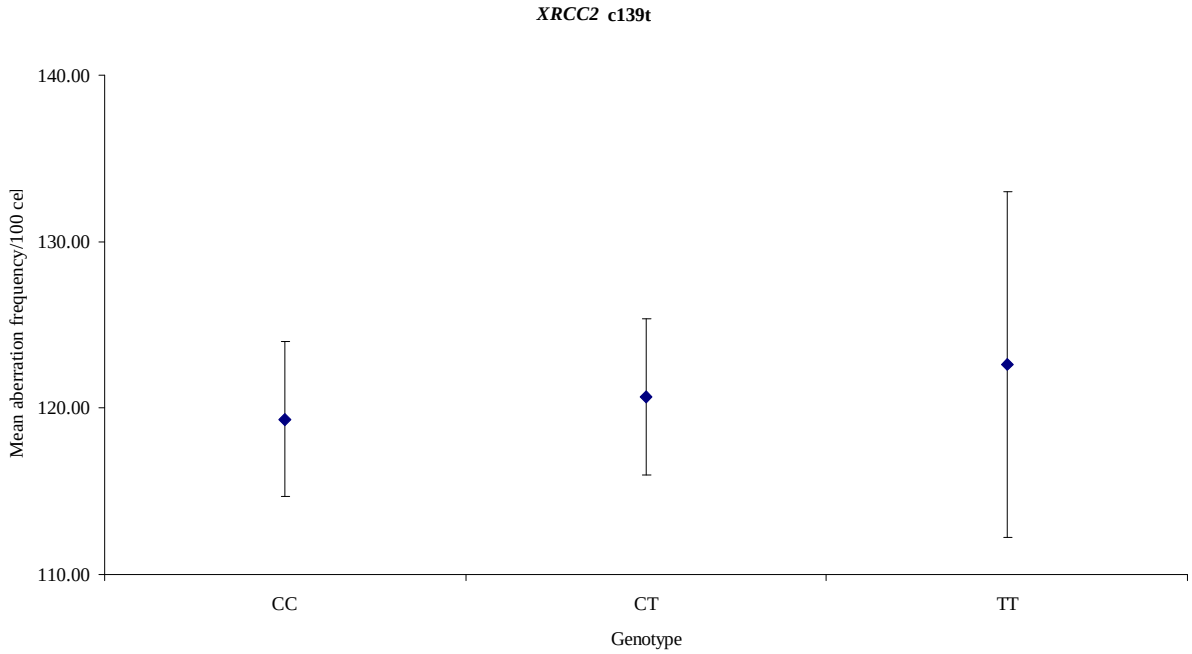


Figure 4.4 j): Association of G_2 radiosensitivity with genotype for XRCC2 c139t.

Wild-type: C / C, wild-type / mutant: C / T, mutant: T / T.

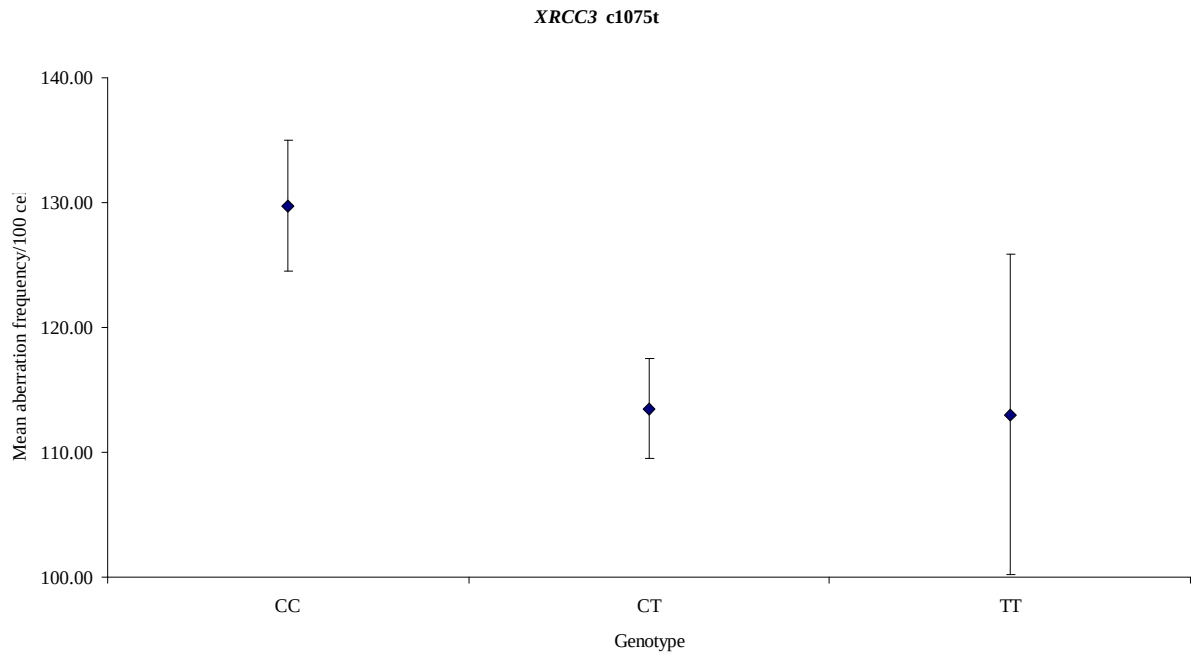


Figure 4.4 k): Association of G_2 radiosensitivity with genotype for XRCC3 c1075t.

Wild-type: C / C, wild-type / mutant: C / T, mutant: T / T.

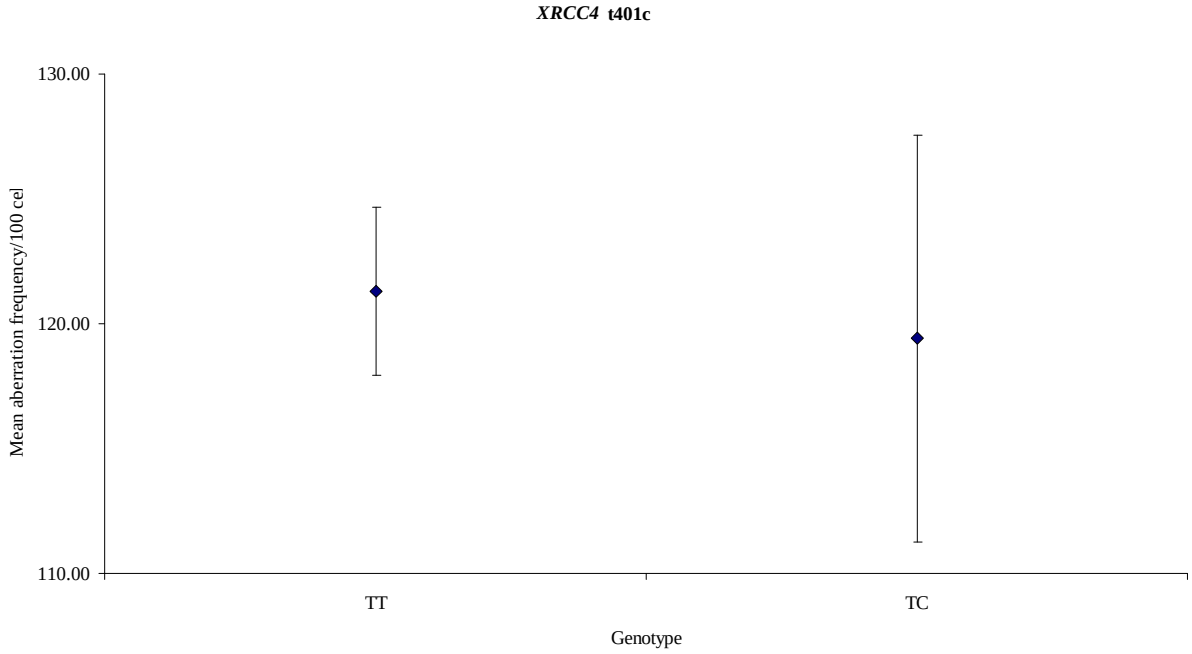


Figure 4.4 l): Association of G_2 radiosensitivity with genotype for XRCC4 t401c.

Wild-type: T / T, wild-type / mutant: T / C, mutant: C / C (non observed).

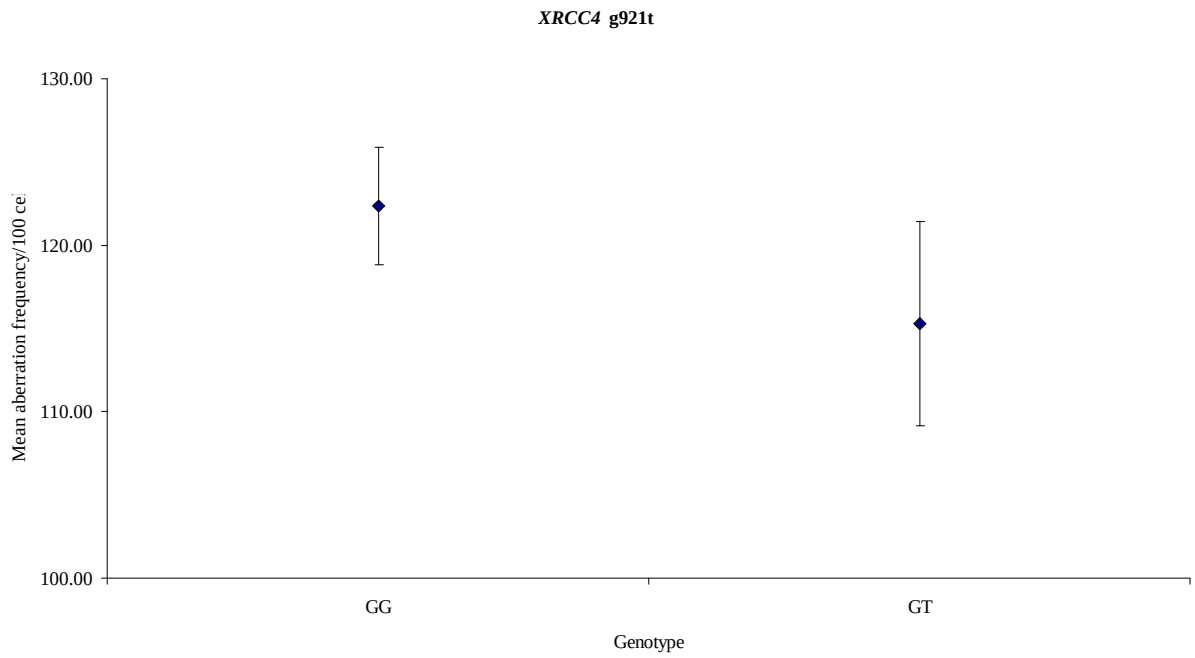


Figure 4.4 m): Association of G_2 radiosensitivity with genotype for XRCC4 g921t.

Wild-type: G / G, wild-type / mutant: G / T, mutant: T / T.

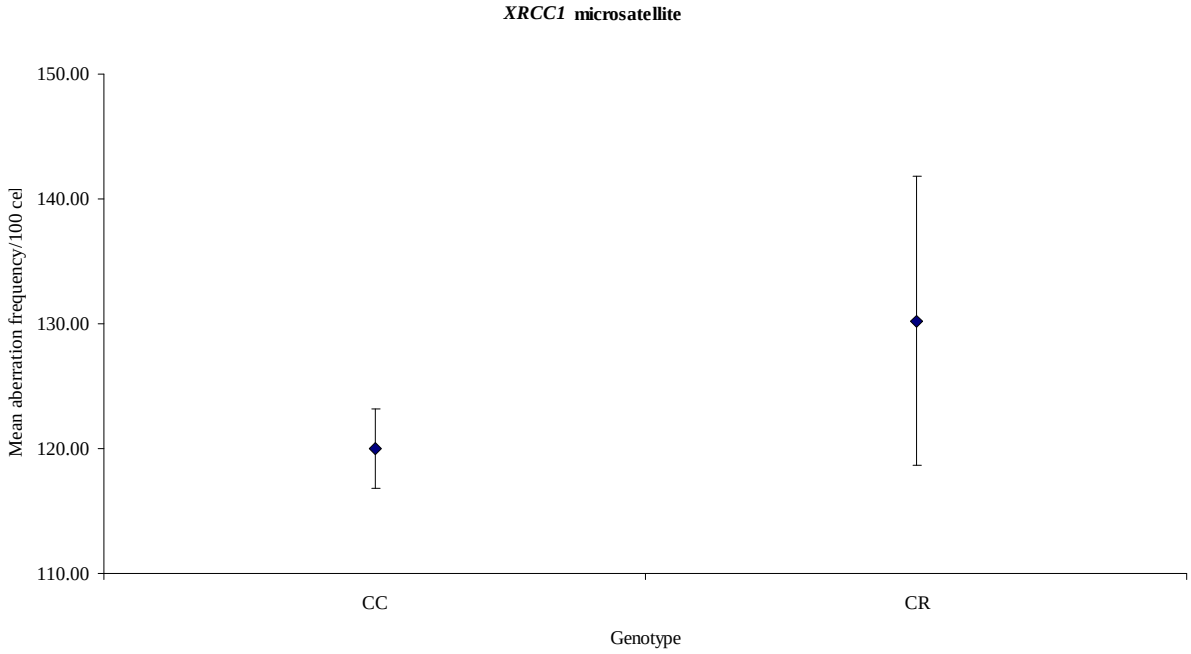


Figure 4.4 n): Association of G_2 radiosensitivity with allele frequencies for XRCC1 microsatellite.

Wild-type: C / C, wild-type / mutant: C / R, mutant: R / R (non observed).

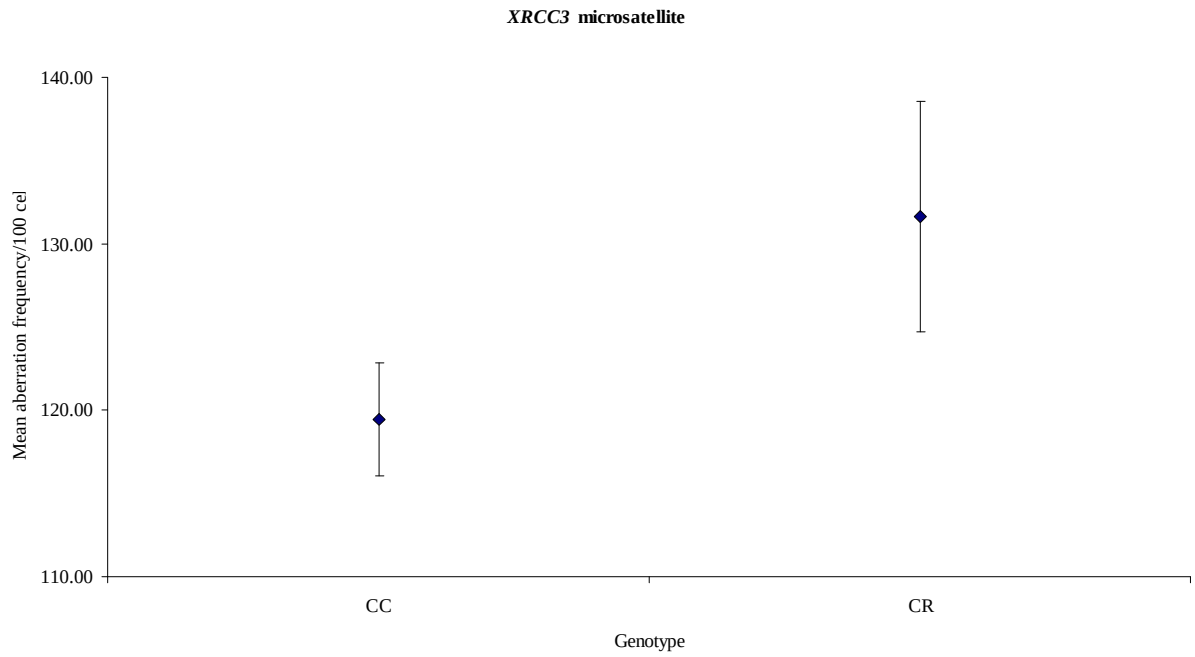


Figure 4.4 o): Association of G_2 radiosensitivity with allele frequencies for XRCC3 microsatellite.

Wild-type: C / C, wild-type / mutant: C / R, mutant: R / R (non observed).

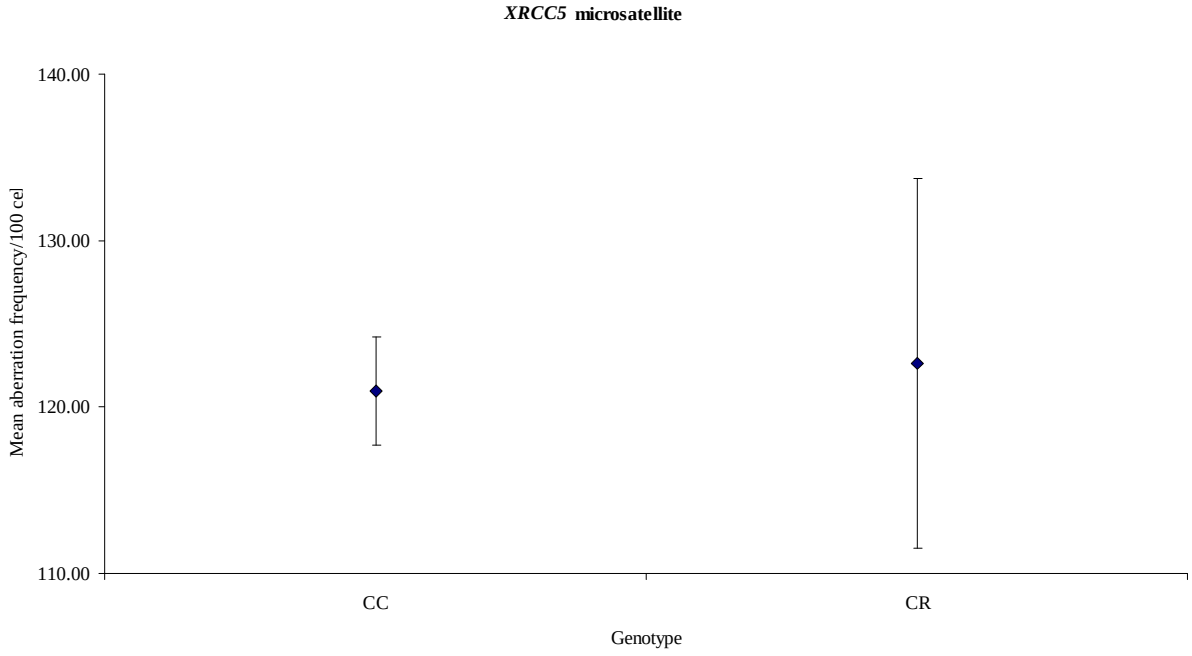


Figure 4.4 p): Association of G_2 radiosensitivity with allele frequencies for XRCC5 microsatellite.

Wild-type: C / C, wild-type / mutant: C / R, mutant: R / R (non observed).

Table 4.14: Summary of the association between G_2 chromosomal radiosensitivity and polymorphisms in DNA repair gene investigated in the survivors of childhood and adolescent cancer, their partners and offspring.

Polymorphism		WT/WT	WT/M	M/M	ANOVA		FBAT	
ADPRT t2444c	Genotype	T/T	T/C	C/C	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	53	26	4				
	Mean aberration yield	125.25	110.92	132.00	6.22	0.076	0.189	0.663
	Standard deviation	28.94	25.45	23.93				
APEX t649g	Genotype	T/T	T/G	G/G	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	15	47	21				
	Mean aberration yield	119.27	123.57	116.81	1.11	0.641	0.016	0.898
	Standard deviation	34.71	26.67	27.57				
hOGG1 c1285g	Genotype	C/C	C/G	G/G	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	52	26	5				
	Mean aberration yield	118.19	128.08	114.80	2.91	0.307	4.254	0.039[1]
	Standard deviation	26.37	31.08	31.71				
hOGG1 g445a	Genotype	G/G	G/A	A/A	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	81	2	0				

	Mean aberration yield	120.98	125.50	-	0.060	0.825	-	-
	Standard deviation	27.96	54.45	-				
XRCC1 c685t	Genotype	C/C	C/T	T/T	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	72	11	0				
	Mean aberration yield	121.79	116.45	-	0.41	0.563	0.132	0.716
	Standard deviation	27.92	31.51	-				
XRCC1 g944a	Genotype	G/G	G/A	A/A	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	70	13	0				
	Mean aberration yield	119.93	127.31	-	0.91	0.391	0.333	0.564
	Standard deviation	28.64	31.74	-				
XRCC1 g1301a	Genotype	G/G	G/A	A/A	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	37	32	14				
	Mean aberration yield	122.00	121.34	118.07	0.24	0.907	0.136	0.712
	Standard deviation	29.46	27.06	29.66				
XRCC4 t401c	Genotype	T/T	T/C	C/C	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	73	10	0				
	Mean aberration yield	121.32	119.40	-	0.05	0.842	1.194	0.274
	Standard deviation	28.75	25.75	-				

XRCC4 g921t	Genotype	G/G	G/T	T/T	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	68	15	0				
	Mean aberration yield	122.37	115.27	-	0.95	0.382	1.398	0.237
	Standard deviation	29.18	23.71	-				
XRCC2 c139t	Genotype	C/C	C/T	T/T	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	35	35	10				
	Mean aberration yield	119.31	120.66	122.60	0.15	0.945	0.004	0.950
	Standard deviation	27.55	27.73	32.80				
XRCC3 c1075t	Genotype	C/C	C/T	T/T	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	39	38	6				
	Mean aberration yield	129.74	113.47	113.00	8.42	0.030	0.335	0.563
	Standard deviation	32.73	24.59	31.40				
XPD g935a	Genotype	G/G	G/A	A/A	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	50	28	5				
	Mean aberration yield	122.48	115.71	137.20	3.36	0.255	0.216	0.642
	Standard deviation	26.81	28.13	41.02				
XPD a2251c	Genotype	A/A	A/C	C/C	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	36	38	9				

	Mean aberration yield	120.47	123.18	114.67	0.84	0.713	0.208	0.648
	Standard deviation	24.81	30.67	32.86				
XRCC1 microsatellite	Genotype	C/C	C/R	R/R	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	74	9	0				
	Mean aberration yield	119.97	130.22	-	1.29	0.307	2.831	0.726
	Standard deviation	27.46	34.72	-				
XRCC3 microsatellite	Genotype	C/C	C/R	R/R	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	72	11	0				
	Mean aberration yield	119.47	131.64	-	2.15	0.185	1.278	0.865
	Standard deviation	28.79	22.99	-				
XRCC5 microsatellite	Genotype	C/C	C/R	R/R	R^2 (%)	*P*	χ^2	*P*
	Number of individuals	78	5	0				
	Mean aberration yield	120.99	122.60	-	0.02	0.902	3.179	0.365
	Standard deviation	22.99	24.87	-				

Table 4.15: Haplotype family based association test for the association of G_2 radiosensitivity with DNA repair gene SNPs.

Gene	Polymorphisms involved	HBAT result	
		χ^2	*P*
hOGG1	c1285g, g445a	5.254	0.072
XRCC1	g1301a, c685t, g944a, microsatellite	2.630	0.757
XRCC4	t401c, g921t	2.592	0.274
XRCC3	c1075t, microsatellite	0.508	0.917
XPD	g935a, a2251c	1.546	0.672

Discussion

Chromosomal radiosensitivity, as measured by the G_2 assay, was investigated in the survivors of childhood and adolescent cancer, their partners and offspring in an attempt to determine the relationship between radiosensitivity and cancer predisposition and whether the cellular response could be inherited. Ultimately, the G_2 assay provides an indirect measure of an individuals capacity to repair DNA damage following X-irradiation of peripheral blood lymphocytes *in vitro.* A number of genes have been identified with both direct and indirect roles in DNA repair (Mohrenweiser *et al.*, 2002). The study reported here attempted to examine for any possible association between candidate polymorphisms in genes involved in the various mammalian DNA repair pathways and G_2 chromosomal radiosensitivity. Overall, no such observations were found although several do warrant further investigation (refer to Figures 4.4: a-p).

Heterogeneity testing

Initial heterogeneity testing of genotype and allele frequencies among childhood and adolescent cancer survivors, their partners and offspring revealed a statistical significance in only one out of the 13 SNPs and three microsatellites analysed. The *APEX* t649g SNP, which results in an aspartic acid (Asp) to glutamic acid (Glu) amino acid change at codon 148 and is involved in the BER pathway, showed a significant difference in genotype frequencies between cancer survivors and their partners but not between parents and their offspring. This difference was due to an enhanced frequency of the wild-type allele (Asp) in the survivors (65%) compared to that of their partners (33%). In a previous study examining the functional significance of several variants of DNA repair genes, in order to determine whether or not they contributed to ionising radiation sensitivity as measured by

prolonged cell cycle G_2 delay and thus elevated cancer risk, a statistical significance between genotype and mitotic delay at the *APEX* t649g SNP site was observed (Hu *et al.*, 2001). Interestingly, individuals with the homozygous mutant genotype were shown to exhibit prolonged delay, in direct dispute with the findings of this study, which reported the *APEX* 148Asp allele to be at an enhanced frequency in cancer survivors. Relative to this, the wild-type allele would imply less mitotic delay based on the findings of Hu *et al.* (2001). Similarly, Scott *et al.* (2003) reported less G_2 arrest in irradiated cells of breast cancer patients than in female controls and hypothesised that for being the reason for their enhanced G_2 chromosomal radiosensitivity, perhaps through a G_2 checkpoint control deficiency. Hu *et al.* (2001) also reported on the *XRCC1* g1301a SNP site, which results in an arginine (Arg) to glutamine (Gln) amino acid change at codon 399, and concluded that the *APEX* 148Glu allele and/or the *XRCC1* 399Gln allele, may interact with family history and contribute to prolonged cell cycle G_2 delay, thus ionising radiation sensitivity and susceptibility to breast cancer. The group also concluded that amino acid substitution variants in BER genes were associated with mitotic delay in response to ionising radiation. In contrast, the current study did not observe any statistical significance in any of the other six SNPs and one microsatellite investigated in genes involved in BER. Likewise, the study of Andreassen *et al.* (2003) found no association between the *APEX* t649g SNP site and the prediction of normal tissue radiosensitivity. Finally, Hu *et al.* (2001) concluded that since inherited sensitivity to ionising radiation and deficient repair of ionising radiation induced DNA damage may serve as markers for low penetrant predisposition genes in breast cancer, then amino acid substitution variants in BER genes may contribute to hereditary ionising radiation sensitivity and cancer susceptibility.

G_2 chromosomal radiosensitivity and polymorphisms in candidate DNA repair genes

On examination of the association between G_2 chromosomal radiosensitivity and polymorphisms in candidate DNA repair genes, ANOVA testing revealed a statistically significant higher mean G_2 score in individuals carrying the *XRCC3* c1075t SNP, which results in a threonine (Thr) to methionine (Met) amino acid substitution at codon 241. Several previous studies have investigated the *XRCC3* c1075t SNP with many of the results suggesting the Met241 variant is deficient in DNA repair (Angelini *et al.*, 2005; Au *et al.*, 2003; Kiuru *et al.*, 2005; Matullo *et al.*, 2001). Angelini *et al*. (2005) studied the frequency of micronuclei (MN), which is an indicator of DNA damage, in a population exposed to low levels of ionising radiaition and in matched controls. The study found that workers with the *XRCC3* variant allele showed a significantly higher MN frequency than controls with the same genotypes. This led to the suggestion that the *XRCC3* polymorphism might contribute to increased genetic damage in susceptible individuals exposed to chronic low levels of ionising radiation. Au *et al*. (2003) investigated the *XRCC3* c1075t SNP site in 80 non-smoking healthy volunteers using the cytogenetic challenge assay (using X-rays) and reported a significant increase in chromosome deletions in the Met241 variant compared with homozygous wild-types. In the study of Kiuru *et al*. (2005) the *XRCC3* homozygous variant genotype was associated with an increase in two-way translocations in a population of 84 healthy nonsmokers. Similarly, Matullo *et al*. (2001) showed the *XRCC3* Met241 variant was significantly associated with higher DNA adduct levels in leukocytes of 133 non-, 93 former and 82 current smokers classed as one group, with Met241 homozygous mutants displaying an average of 11.4 ± 1.5 adducts/10^9 nucleotides in comparison with 7.7 ± 0.9 and 6.9 ± 1.1 adducts/10^9 nucleotides in Thr/Met heterozygotes and Thr/Thr homozygotes, respectively. Since the process of smoking may have compromised the DNA repair capacity of leukocytes in

smokers, non-smokers alone were examined and also showed a significant association. In contrast, the variant allele was not found to be significantly associated with lung carcinoma risk (David-Beabes *et al.*, 2001) or bladder cancer risk (Stern *et al.*, 2002), although for the latter, heavy smokers with the variant Met allele had about twice the risk of those without it, and finally, the results of Tuimala *et al.* (2004) did not indicate any association of the *XRCC3* polymorphism with the level of sister chromatid exchanges in a group of 60 healthy Caucasians. The findings reported in the current study do not provide further evidence to support the many observations of deficient DNA repair associated with the *XRCC3* Met241 variant that have previously been reported. The results indicated that individuals with the wild-type homozygous genotype displayed a higher mean G_2 score (129.74 ± 5.74) compared to those carrying one Met allele (113.47 ±3.99), leading to the suggestion that the *XRCC3* c1075t SNP is influential in the DNA repair process but rather that it is the Thr allele which confers less efficient repair. However, this result failed to meet the stringency of significance following multiple test adjustment. In addition, FBAT analysis (refer to page 149) failed to reveal a significant departure from the no linkage and no association hypothesis and haplotype-based FBAT analyses of the *XRCC3* c1075t SNP and *XRCC3* microsatellite also failed to detect an association, leaving the finding suggestive rather than conclusive.

In terms of FBAT analyses, only the *hOGG1* c1285g SNP site revealed an association with G_2 score although this was a rather weak relationship and failed to remain significant following relevant adjustments for multiple testing. Moreover, haplotype-based FBAT analyses of the *hOGG1* c1285g and *hOGG1* g445a SNP sites did not detect any relationship with chromosomal radiosensitivity, although this was marginally non-significant (P = 0.072). Regardless of this, other studies have reported poor DNA repair

associated with the *hOGG1* c1285g variant, which results in a serine (Ser) to cysteine (Cys) amino acid change at codon 326. Yarosh *et al.* (2005) correlated DNA repair activity in three breast cancer cell lines each with differing genotypes (homozygous wild-type, heterozygous and homozygous variant) and found a significant deficiency in repair in the Cys326 homozygous mutant cell line. The variant allele has also been associated with an increased risk of several types of cancer including lung, prostate, nasopharyngeal and oesophageal (Cho *et al.*, 2003; Goode *et al.*, 2002). In contrast, De Ruyck *et al.* (2005) failed to detect any relationship between the Cys326 variant allele and an increased risk of gynaecological tumours (cervical and endometrial cancer) and Goode *et al.* (2002) reported on a Brazilian stomach cancer study that also revealed no association.

To date, only one other study has directly investigated the relationship between chromosomal radiosensitivity as measured by the G_2 assay and polymorphisms in DNA repair genes, namely the *XPD* gene polymorphisms (Lunn *et al.*, 2000). The study of De Ruyck *et al.* (2005) examined the association of several polymorphisms in the *XRCC1*, *XRCC3* and *hOGG1* genes with the development of late radiotherapy reactions and assessed the correlation between *in vitro* G_2 chromosomal radiosensitivity and clinical radiosensitivity but did not directly correlate G_2 score and genotype. In the Lunn *et al.* (2000) study, three SNP sites in *XPD* were studied, these being a2251c, g935a and c598g which result in an amino acid change of lysine (Lys) to glutamine (Gln) at codon 751, aspartic acid (Asp) to asparagine (Asn) at codon 312 and isoleucine (Ile) to methionine (Met) at codon 199, respectively. The results reported that individuals homozygous for the wild-type Lys751 allele had significantly higher chromatid aberrations than those having a Gln751 allele, suggesting that the Lys751 allele may alter the XPD protein product resulting in an increased risk of sub-optimal DNA repair in these individuals. In addition,

individuals homozygous for the wild-type Asp312 allele had higher levels of chromatid aberrations than those having at least one variant Asn312 allele. However, this was not statistically significant. No variants were found at codon 199 precluding its further analysis in the study. In the current study, both the *XPD* a2251c and g935a polymorphisms were studied. None of the analyses performed (ANOVA, FBAT or haplotype-based FBAT) revealed any type of association to suggest that either of the polymorphisms investigated in the *XPD* gene could be correlated to G_2 sensitivity, therefore failing to confirm the findings of Lunn *et al.* (2000). However, the present findings do provide support for other studies which have also failed to reveal any association between *XPD* gene polymorphisms and DNA repair capacity (David-Beabes *et al.*, 2001; Duell *et al.*, 2000). In the study by Duell *et al*. (2000) no association was found between the Lys751Gln polymorphism and DNA repair efficiency as measured by sister chromatid exchange frequencies and polyphenol DNA adducts in lymphocytes. In addition, David-Beabes *et al.* (2001) found no association between lung cancer risk and the Lys751Gln polymorphism. In contrast, Au *et al.* (2003) showed a significant increase in UV-induced chromosome aberrations, using the cytogenetic challenge assay in lymphocytes, for the homozygous variant genotypes in both *XPD* SNP sites investigated, indicating insufficient repair of UV-induced DNA damage, i.e. NER deficiency. However, variant genotypes had no significant effect on chromosome damage following exposure to X-rays, confirming XPD is not involved in BER to any great extent. The findings of Au *et al.* (2003) may suggest that the earlier studies mentioned (Duell *et al.*, 2000; Lunn *et al.*, 2000) and indeed the present study (through the use of X-rays to induce DNA damage rather than UV radiation), are not the relevant biomarkers for *XPD* variant genotypes in the NER pathway.

Despite the fact that no definite associations between G_2 chromosomal radiosensitivity and polymorphisms in DNA repair genes have been elucidated in this study, a number of reasons exist to warrant an extension of this preliminary work, both in terms of the polymorphisms studied and sample size. Small sample numbers often fail to provide the statistical power needed to detect such associations in a study of this type. Increasing donor numbers and the range of polymorphisms investigated within DNA repair genes may alleviate this and actually show if the putative associations observed here are actually real. For example, the near non-significance of the haplotype-based FBAT results for the *hOGG1* gene is worth further investigation, since with a larger sample size this is likely to show significance if this association is indeed valid. Furthermore, the heterogeneity and types of cancers analysed in this study may have influenced the likelihood of any possible associations between G_2 chromosomal radiosensitivity and polymorphisms in DNA repair gene, as discussed in Chapter 3. Concentrating on specific cancer types and cancers that have a definite DNA damage repair defect may be a source of more useful information when trying to determine an association with G_2 chromosomal radiosensitivity or identify low penetrance susceptibility genes.

Conclusions

There is substantial scientific literature available examining polymorphisms in candidate DNA repair genes, using various techniques to measure repair efficacy and thus highlighting the interest in the hypothesis that polymorphisms in DNA repair genes reduce the capacity of the protein product to repair DNA damage and thus lead to an increased cancer susceptibility. Much of the research is focused on experiments attempting to measure a functional effect of these polymorphisms, whilst in truth, no unequivocal evidence for an altered repair capacity actually exists. In addition, many SNP association

studies are not applicable to the disease being investigated. Nevertheless, if G_2 chromosomal radiosensitivity is a marker of low penetrance cancer susceptibility genes involved in coping with damage to the genome, then appropriate methods must be sought to identify them. However, low penetrance alleles only confer a degree of susceptibility to a particular phenotype and thus fail to exhibit traditional Mendelian inheritance. Haplotype-based analyses and high-throughput SNP genotyping are professed to be the way forward in this area, providing the statistical power needed to detect disease associations. Such analyses could potentially identify unknown non-functional SNPs within the linkage disequilibrium of already identified functionally involved polymorphisms. Moreover, haplotype information will facilitate in the analysis of multiple genes within the same DNA repair pathways and thus provide a more comprehensive insight into the associations studied. In any event, it is now universally accepted that the identification of low penetrance genes that predispose to common cancers will involve a large number of alleles from various pathways, each conferring a small genotypic risk. The unravelling of the human genome, along with continued advances in SNP technology and high-throughput genotyping methods can only serve to identify these risks. In terms of the potential of G_2 chromosomal radiosensitivity/SNP association studies, there may be value in measuring sensitivity using the G_2 assay as a pre-molecular analysis screening tool, serving to identify individuals from normal populations who are likely to carry many low penetrance cancer predisposition gene mutations. SNP genotyping could then be performed to identify a radiosensitive genotype through comparison with control individuals exhibiting a low G_2 score.

REFERENCES

Aka P, Mateuca R, Buchet JP, Thierens H, Kirsch-Volders M (2004) Are genetic polymorphisms in OGG1, XRCC1 and XRCC3 genes predictive for the DNA strand break repair phenotype and genotoxicity in workers exposed to low dose ionising radiations? *Mutation Research* **556:** 169-181.

Almasy L, Blangero J (1998) Multipoint quantitative-trait linkage analysis in general pedigrees. *American Journal of Human Genetics* **62:** 1198-1211.

Allen C, Halbrook J, Nickoloff JA (2003) Interactive competition between homologous recombination and non-homologous end joining. *Molecular Cancer Research* **1:** 913-920.

Andreassen CN, Alsner J, Overgaard M, Overgaard J (2003) Prediction of normal tissue radiosensitivity from polymorphisms in candidate genes. *Radiotherapy and Oncology* **69:** 127-135.

Angelini S, Kumar R, Carbone F, Maffei F, Forti GC, Violante FS, Lodi V, Curti S, Hemminki K, Hrelia P (2005) Micronuclei in humans induced by exposure to low level of ionizing radiation: influence of polymorphisms in DNA repair genes. *Mutation Research* **570:** 105-117.

Arlett CF (1986) Human DNA repair defects. *Journal of Inherited Metabolic Disease* **9 Suppl 1:** 69-84.

Arlett CF, Harcourt SA (1980) Survey of radiosensitivity in a variety of human cell strains. *Cancer Research* **40:** 926-932.

Arlett CF, Priestley A (1985) An assessment of the radiosensitivity of ataxia-telangiectasia heterozygotes. *Kroc Foundation Series* **19:** 101-109.

Au WW, Salama SA, Sierra-Torres CH (2003) Functional characterization of polymorphisms in DNA repair genes using cytogenetic challenge assays. *Environmental Health Perspectives* **111:** 1843-1850.

Aurias A, Antoine JL, Assathiany R, Odievre M, Dutrillaux B (1985) Radiation sensitivity of Bloom's syndrome lymphocytes during S and G2 phases. *Cancer Genetics and Cytogenetics* **16:** 131-136.

Baeyens A, Thierens H, Claes K, Poppe B, Messiaen L, De Ridder L, Vral A (2002) Chromosomal radiosensitivity in breast cancer patients with a known or putative genetic predisposition. *British Journal of Cancer* **87:** 1379-1385.

Baeyens A, Vandersickel V, Thierens H, Ridder LD, Vral A (2005) Effects of estradiol and progesterone on the variability of the micronucleus assay. *Mutation Research* **578:** 308-316.

Baria K, Warren C, Eden OB, Roberts SA, West CM, Scott D (2002) Chromosomal radiosensitivity in young cancer patients: possible evidence of genetic predisposition. *International Journal of Radiation Biology* **78:** 341-346.

Baria K, Warren C, Roberts SA, West CM, Scott D (2001) Chromosomal radiosensitivity as a marker of predisposition to common cancers? *British Journal of Cancer* **84:** 892-896.

Baria K, Warren C, Scott D (1999) Chromosomal radiosensitivity and cancer predisposition. *British Journal of Cancer* **80:** P37.

Beamish H, Lavin MF (1994) Radiosensitivity in ataxia-telangiectasia: anomalies in radiation-induced cell cycle delay. *International Journal of Radiation Biology* **65:** 175-184.

Bender MA, Rary JM, Kale RP (1985) G2 chromosomal radiosensitivity in ataxia telangiectasia lymphocytes. *Mutation Research* **152:** 39-47.

Bender MA, Viola MV, Fiore J, Thompson MH, Leonard RC (1988) Normal G2 chromosomal radiosensitivity and cell survival in the cancer family syndrome. *Cancer Research* **48:** 2579-2584.

Biade S, Stobbe CC, Chapman JD (1997) The intrinsic radiosensitivity of some human tumor cells throughout their cell cycles. *Radiation Research* **147:** 416-421.

Bigelow SB, Rary JM,Bender MA (1979) G2 chromosomal radiosensitivity in Fanconi's anemia. *Mutation Research* **63:** 189-199.

Bryant PE (1998a) Mechanisms of radiation-induced chromatid breaks. *Mutation Research* **404:** 107-111.

Bryant PE (1998b) The signal model: a possible explanation for the conversion of DNA double-strand breaks into chromatid breaks. *International Journal of Radiation Biology* **73:** 243-251.

Bryant PE, Gray L, Riches AC, Steel CM, Finnon P, Howe O, Kesterton I, Vral A, Curwen GB, Smart V, Tawn EJ, Whitehouse CA (2002) The G2 chromosomal radiosensitivity assay. *International Journal of Radiation Biology* **78:** 863-866.

Bryant PE, Gray LJ, Peresse N (2004) Progress towards understanding the nature of chromatid breakage. *Cytogenetic and Genome Research* **104:** 65-71.

Chandler ME, Yunis JJ (1978) A high resolution in situ hybridization technique for the direct visualization of labeled G-banded early metaphase and prophase chromosomes. *Cytogenetics and Cell Genetics* **22:** 352-356.

Cho E-Y, Hildesheim A, Chen C-J, Hsu M-M, Chen I-H, Mittl BF, Levine PH, Liu M-Y, Chen J-Y, Brinton LA, Cheng Y-J, Yang C-S (2003) Nasopharyngeal carcinoma and genetic polymorphisms of DNA repair enzymes XRCC1 and hOGG1. *Cancer Epidemiology Biomarkers and Prevention* **12:** 1100-1104.

Cleaver JE (1968) Defective repair replication of DNA in xeroderma pigmentosum. *Nature* **218:** 652-656.

Cottet F, Blanche H, Verasdonck P, Le Gall I, Schachter F, Burkle A, Muiras ML (2000) New polymorphisms in the human poly(ADP-ribose) polymerase-1 coding sequence: lack

of association with longevity or with increased cellular poly(ADP-ribosyl)ation capacity. *Journal of Molecular Medicine* **78:** 431-440.

Curwen GB, Winther JF, Tawn EJ, Smart V, Whitehouse CA, Rees GS, Olsen JH, Guldberg P, Rechnitzer C, Schroder H, Bryant PE, Sheng X, Lee HS, Chakraborty R, Boice JD (2005) G(2) chromosomal radiosensitivity in Danish survivors of childhood and adolescent cancer and their offspring. *British Journal of Cancer* **93:** 1038-1045.

Darroudi F, Vyas RC, Vermeulen S, Natarajan AT (1995) G2 radiosensitivity of cells derived from cancer-prone individuals. *Mutation Research* **328:** 83-90.

David-Beabes GL, Lunn RM, London SJ (2001) No association between the XPD (Lys751G1n) polymorphism or the XRCC3 (Thr241Met) polymorphism and lung cancer risk. *Cancer Epidemiology Biomarkers and Prevention* **10:** 911-912.

De la Vega FM, Lazaruk KD, Rhodes MD, Wenz MH (2005) Assessment of two flexible and compatible SNP genotyping platforms: TaqMan SNP Genotyping Assays and the SNPlex Genotyping System. *Mutation Research* **573:** 111-135.

De Ruyck K, Van Eijkeren M, Claes K, Morthier R, De Paepe A, Vral A, De Ridder L, Thierens H (2005) Radiation-induced damage to normal tissues after radiotherapy in patients treated for gynecologic tumors: Association with single nucleotide polymorphisms in XRCC1, XRCC3, and OGG1 genes and in vitro chromosomal radiosensitivity in lymphocytes. *International Journal of Radiation Oncology*Biology*Physics* **62:** 1140-1149.

Duell EJ, Wiencke JK, Cheng TJ, Varkonyi A, Zuo ZF, Ashok TD, Mark EJ, Wain JC, Christiani DC, Kelsey KT (2000) Polymorphisms in the DNA repair genes XRCC1 and ERCC2 and biomarkers of DNA damage in human blood mononuclear cells. *Carcinogenesis* **21:** 965-971.

Easton DF (1994) Cancer risks in A-T heterozygotes. *International Journal of Radiation Biology* **66:** S177-182.

Falck J, Petrini JH, Williams BR, Lukas J, Bartek J (2002) The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. *Nature Genetics* **30:** 290-294.

Fenech M (1999) Micronucleus frequency in human lymphocytes is related to plasma vitamin B12 and homocysteine. *Mutation Research* **428:** 299-304.

Ford BN, Ruttan CC, Kyle VL, Brackley ME, Glickman BW (2000) Identification of single nucleotide polymorphisms in human DNA repair genes. *Carcinogenesis* **21:** 1977-1981.

Franchitto A, Pichierri P, Genuardi M, De Santis A, Palitti F (2001) Investigation of G2-phase chromosomal radiosensitivity in hereditary non-polyposis colorectal cancer cells. *International Journal of Radiation Biology* **77:** 773-780.

Gatti RA, Berkel I, Boder E, Braedt G, Charmley P, Concannon P, Ersoy F, Foroud T, Jaspers NG, Lange K *et al*. (1988) Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. *Nature* **336:** 577-580.

Godderis L, De Boeck M, Haufroid V, Emmery M, Mateuca R, Gardinal S, Kirsch-Volders M, Veulemans H, Lison D (2004) Influence of genetic polymorphisms on biomarkers of exposure and genotoxic effects in styrene-exposed workers. *Environmental and Molecular Mutagenesis* **44:** 293-303.

Goode EL, Ulrich CM, Potter JD (2002) Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiology Biomarkers and Prevention* **11:** 1513-1530.

Griffin CS, Harvey AN, Savage JR (1994) Chromatid damage induced by 238Pu alpha-particles in G2 and S phase Chinese hamster V79 cells. *International Journal of Radiation Biology* **66:** 85-98.

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* **48:** 361-372. Abstract only.

Gutierrez-Enriquez S, Fernet M, Dork T, Bremer M, Lauge A, Stoppa-Lyonnet D, Moullan N, Angele S, Hall J (2004) Functional consequences of ATM sequence variants for chromosomal radiosensitivity. *Genes Chromosomes and Cancer* **40:** 109-119.

Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* **246:** 629-634.

Heddle JA, Bodycote DJ (1970) On the formation of chromosomal aberrations. *Mutation Research* **9:** 117-126.

Heddle JA, Whissell D, Bodycote DJ (1969) Changes in chromosome structure induced by radiations: a test of the two chief hypotheses. *Nature* **221:** 1158-1160.

Hemminki K, Bermejo JL (2005) Relationships between familial risks of cancer and the effects of heritable genes and their SNP variants. *Mutation Research* **592:** 6-17.

Hemminki K, Forsti A, Bermejo JL (2005) Single nucleotide polymorphisms (SNPs) are inherited from parents and they measure heritable events. *Journal of Carcinogenesis* **4:** 2.

Hickson ID, Harris AL (1988) Mammalian DNA repair--use of mutants hypersensitive to cytotoxic agents. *Trends in Genetics* **4:** 101-106.

Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411:** 366-374.

Horvath S, Xu X, Laird NM (2001) The family based association test method: strategies for studying general genotype--phenotype associations. *European Journal of Human Genetics* **9:** 301-306.

Howard A, Pelc, SR (1953) Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Hereditary* **Suppl 6:** 261.

Howe O, Daly P, Seymour C, Ormiston W, Nolan C, Mothersill C (2005) Elevated G2 chromosomal radiosensitivity in Irish breast cancer patients: a comparison with other studies. *International Journal of Radiation Biology* **81:** 373-378.

Hsu TC (1983) Genetic instability in the human population: a working hypothesis. *Hereditas* **98:** 1-9.

Hu JJ, Smith TR, Miller MS, Mohrenweiser HW, Golden A, Case LD (2001) Amino acid substitution variants of APE1 and XRCC1 genes associated with ionizing radiation sensitivity. *Carcinogenesis* **22:** 917-922.

International Commission on Radiological Protection, ICRP publication 79 (1998) Genetic susceptibility to cancer. *Annals of the ICRP* **28:** 1-157. Approved by the Commission in May 1997.

Jackson SP (2002) Sensing and repairing DNA double-strand breaks. *Carcinogenesis* **23:** 687-696.

Jaspers NG, Gatti RA, Baan C, Linssen PC, Bootsma D (1988) Genetic complementation analysis of ataxia telangiectasia and Nijmegen breakage syndrome: a survey of 50 patients. *Cytogenetics and Cell Genetics* **49:** 259-263.

Jaspers NG, Painter RB, Paterson MC, Kidson C, Inoue T (1985) Complementation analysis of ataxia-telangiectasia. *Kroc Foundation Series* **19:** 147-162.

Kanda R, Hayata I (1999) Effect of estradiol on radiation-induced chromosome aberrations in human lymphocytes. *Journal of Radiation Research* **40:** 95-100.

Kastan MB, Lim DS (2000) The many substrates and functions of ATM. *Nature Reviews*

Molecular Cell Biology **1:** 179-186.

Kerr B, Ashcroft GS, Scott D, Horan MA, Ferguson MW, Donnai D (1996) Rothmund-Thomson syndrome: two case reports show heterogeneous cutaneous abnormalities, an association with genetically programmed ageing changes, and increased chromosomal radiosensitivity. *Journal of Medical Genetics* **33:** 928-934.

Kiuru A, Lindholm C, Heilimo I, Ceppi M, Koivistoinen A, Ilus T, Hirvonen A, Norppa H, Salomaa S (2005) Influence of DNA repair gene polymorphisms on the yield of chromosomal aberrations. *Environmental and Molecular Mutagenesis* **46:** 198-205.

Kuhn EM (1980) Effects of X-irradiation in G1 and G2 on Bloom's Syndrome and normal chromosomes. *Human Genetics* **54:** 335-341.

Kuhne M, Riballo E, Rief N, Rothkamm K, Jeggo PA, Lobrich M (2004) A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. *Cancer Research* **64:** 500-508.

Li CC (1976) *First Course in Population Genetics*. Boxwood Press: Pacific Grove, California.

Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K (2000) Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *New England Journal of Medicine* **343:** 78-85.

Lunn RM, Helzlsouer KJ, Parshad R, Umbach DM, Harris EL, Sanford KK, Bell DA (2000) XPD polymorphisms: effects on DNA repair proficiency. *Carcinogenesis* **21:** 551-555.

Mack TM, Cozen W, Shibata DK, Weiss LM, Nathwani BN, Hernandez AM, Taylor CR, Hamilton AS, Deapen DM, Rappaport EB (1995) Concordance for Hodgkin's disease in identical twins suggesting genetic susceptibility to the young-adult form of the disease. *New England Journal of Medicine* **332:** 413-418.

Marcon F, Andreoli C, Rossi S, Verdina A, Galati R, Crebelli R (2003) Assessment of individual sensitivity to ionizing radiation and DNA repair efficiency in a healthy population. *Mutation Research* **541:** 1-8.

Marsh D, Zori R (2002) Genetic insights into familial cancers-- update and recent discoveries. *Cancer Letters* **181:** 125-164.

Matullo G, Palli D, Peluso M, Guarrera S, Carturan S, Celentano E, Krogh V, Munnia A, Tumino R, Polidoro S, Piazza A, Vineis P (2001) XRCC1, XRCC3, XPD gene polymorphisms, smoking and (32)P-DNA adducts in a sample of healthy subjects. *Carcinogenesis* **22:** 1437-1445.

Mitchell EL, Scott D (1997) G2 chromosomal radiosensitivity in fibroblasts of ataxia-telangiectasia heterozygotes and a Li-Fraumeni syndrome patient with radioresistant cells. *International Journal of Radiation Biology* **72:** 435-438.

Mohrenweiser HW, Jones IM (1998) Variation in DNA repair is a factor in cancer susceptibility: a paradigm for the promises and perils of individual and population risk estimation? *Mutation Research* **400:** 15-24.

Mohrenweiser HW, Wilson DM, Jones IM (2003) Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. *Mutation Research* **526:** 93-125.

Mohrenweiser HW, Xi T, Vazquez-Matias J, Jones IM (2002) Identification of 127 amino acid substitution variants in screening 37 DNA repair genes in humans. *Cancer Epidemiology Biomarkers and Prevention* **11:** 1054-1064.

Montesano R, Hall J (2001) Environmental causes of human cancers. *European Journal of Cancer* **37 Suppl 8:** S67-87.

Mozdarani H, Bryant PE (1989) Kinetics of chromatid aberrations in G2 ataxia-telangiectasia cells exposed to X-rays and ara A. *International Journal of Radiation Biology* **55:** 71-84.

Nagy R, Sweet K, Eng C (2004) Highly penetrant hereditary cancer syndromes. *Oncogene* **23:** 6445-6470.

Natarajan AT, Meyers M (1979) Chromosomal radiosensitivity of ataxia telangiectasia cells at different cell cycle stages. *Human Genetics* **52:** 127-132.

Natarajan AT, Obe G (1978) Molecular mechanisms involved in the production of chromosomal aberrations. I. Utilization of Neurospora endonuclease for the study of aberration production in G2 stage of the cell cycle. *Mutation Research* **52:** 137-149.

Neubauer S, Arutyunyan R, Stumm M, Dork T, Bendix R, Bremer M, Varon R, Sauer R, Gebhart E (2002) Radiosensitivity of ataxia telangiectasia and Nijmegen breakage syndrome homozygotes and heterozygotes as determined by three-color FISH chromosome painting. *Radiation Research* **157:** 312-321.

Obe G, Gobel D, Engeln H, Herha J, Natarajan AT (1980) Chromosomal aberrations in peripheral lymphocytes of alcoholics. *Mutation Research* **73:** 377-386.

Pandita TK, Hittelman WN (1992a) The contribution of DNA and chromosome repair deficiencies to the radiosensitivity of ataxia-telangiectasia. *Radiation Research* **131:** 214-223.

Pandita TK, Hittelman WN (1992b) Initial chromosome damage but not DNA damage is greater in ataxia telangiectasia cells. *Radiation Research* **130:** 94-103.

Pandita TK, Hittelman WN (1995) Evidence of a chromatin basis for increased mutagen sensitivity associated with multiple primary malignancies of the head and neck. *International Journal of Cancer* **61:** 738-743.

Papworth R, Slevin N, Roberts SA, Scott D (2001) Sensitivity to radiation-induced chromosome damage may be a marker of genetic predisposition in young head and neck

cancer patients. *British Journal of Cancer* **84:** 776-782.

Parshad R, Price FM, Bohr VA, Cowans KH, Zujewski JA, Sanford KK (1996) Deficient DNA repair capacity, a predisposing factor in breast cancer. *British Journal of Cancer* **74:** 1-5.

Parshad R, Price FM, Pirollo KF, Chang EH, Sanford KK (1993a) Cytogenetic response to G2-phase X irradiation in relation to DNA repair and radiosensitivity in a cancer-prone family with Li-Fraumeni syndrome. *Radiation Research* **136:** 236-240.

Parshad R, Sanford KK, Jones GM (1983) Chromatid damage after G2 phase x-irradiation of cells from cancer-prone individuals implicates deficiency in DNA repair. *Proceedings of the National Academy of Sciences of the United States of America* **80:** 5612-5616.

Parshad R, Sanford KK, Jones GM, Tarone RE (1985) G2 chromosomal radiosensitivity of ataxia-telangiectasia heterozygotes. *Cancer Genetics and Cytogenetics* **14:** 163-168.

Parshad R, Sanford KK, Kraemer KH, Jones GM, Tarone RE (1990) Carrier detection in xeroderma pigmentosum. *Journal of Clinical Investigations* **85:** 135-138.

Parshad R, Tarone RE, Price FM, Sanford KK (1993b) Cytogenetic evidence for differences in DNA incision activity in xeroderma pigmentosum group A, C and D cells after X-irradiation during G2 phase. *Mutation Research* **294:** 149-155.

Patel RK, Trivedi AH, Arora DC, Bhatavdekar JM, Patel DD (1997) DNA repair

proficiency in breast cancer patients and their first-degree relatives. *International Journal of Cancer* **73:** 20-24.

Peto J, Houlston RS (2001) Genetics and the common cancers. *European Journal of Cancer* **37 Suppl 8:** S88-96.

Price EA, Bourne SL, Radbourne R, Lawton PA, Lamerdin J, Thompson LH, Arrand JE (1997) Rare microsatellite polymorphisms in the DNA repair genes XRCC1, XRCC3 and XRCC5 associated with cancer in patients of varying radiosensitivity. *Somatic Cell and Molecular Genetics* **23:** 237-247.

Price FM, Parshad R, Tarone RE, Sanford KK (1991) Radiation-induced chromatid aberrations in Cockayne syndrome and xeroderma pigmentosum group C fibroblasts in relation to cancer predisposition. *Cancer Genetics and Cytogenetics* **57:** 1-10.

Rees GS, Trikic MZ, Winther JF, Tawn EJ, Stovall M, Olsen JH, Rechnitzer C, Schroder H, Guldberg P, Boice JD, Jr. (2006) A pilot study examining germline minisatellite mutations in the offspring of Danish childhood and adolescent cancer survivors treated with radiotherapy. *International Journal of Radiation Biology* **82:** 153-160.

Revell SH (1955) A new hypothesis for 'chromatid' changes. *Proceedings of the Radiobiology Symposium*: 243-253. Edited by Bacq SH and Alexander P (Butterworth, London, 1954).

Revell SH (1959) The accurate estimation of chromatid breakage, and its relevance to a

new interpretation of chromatid aberrations induced by ionizing radiations. *Proceedings of the Royal Society of London. Series B, Papers of Biological Science* **150:** 563-589.

Riches AC, Bryant PE, Steel CM, Gleig A, Robertson AJ, Preece PE, Thompson AM (2001) Chromosomal radiosensitivity in G2-phase lymphocytes identifies breast cancer patients with distinctive tumour characteristics. *British Journal of Cancer* **85:** 1157-1161.

Ricoul M, Dutrillaux B (1991) Variations of chromosome radiation sensitivity in fetal and adult mice during gestation. *Mutation Research* **250:** 331-335.

Ricoul M, Sabatier L, Dutrillaux B (1997) Increased chromosome radiosensitivity during pregnancy. *Mutation Research* **374:** 73-78.

Roberts CJ, Morgan GR, Danford N (1997) Effect of hormones on the variation of radiosensitivity in females as measured by induction of chromosomal aberrations. *Environmental Health Perspectives* **105 Suppl 6:** 1467-1471.

Roberts SA, Levine EL, Scott D (2003) Influence of intrinsic radiosensitivity on the survival of breast cancer patients. *International Journal of Radiation Biology* **79:** 311-317.

Roberts SA, Spreadborough AR, Bulman B, Barber JB, Evans DG, Scott D (1999) Heritability of cellular radiosensitivity: a marker of low-penetrance predisposition genes in breast cancer? *American Journal of Human Genetics* **65:** 784-794.

Roff DA, Bentzen P (1989) The statistical analysis of mitochondrial DNA polymorphisms:

chi 2 and the problem of small samples. *Molecular Biology and Evolution* **6:** 539-545.

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of Biological Chemistry* **273:** 5858-5868.

Rooney DE (2001) Protocol 10: Slide preparation. *Human Cytogenetics: malignancy and acquired abnormalities*, Rooney, D.E. (ed) 14-15. PAS. OXFORD.

SAGE (2004) *Statistical Analysis for Genetic Epidemiology* Releasr 5.0.

Sanford KK, Parshad R, Gantt R, Tarone RE, Jones GM, Price FM (1989) Factors affecting and significance of G2 chromatin radiosensitivity in predisposition to cancer. *International Journal of Radiation Biology* **55:** 963-981.

Sanford KK, Parshad R, Price FM, Jones GM, Tarone RE, Eierman L, Hale P, Waldmann TA (1990) Enhanced chromatid damage in blood lymphocytes after G2 phase x irradiation, a marker of the ataxia-telangiectasia gene. *Journal of the National Cancer Institute* **82:** 1050-1054.

Sanford KK, Parshad R, Price FM, Tarone RE, Benedict WF (1996) Cytogenetic responses to G2 phase x-irradiation of cells from retinoblastoma patients. *Cancer Genetics and Cytogenetics* **88:** 43-48.

Sanford KK, Parshad R, Price FM, Tarone RE, Lehmann AR (1995) G2 phase repair of X-

ray-induced chromosomal DNA damage in trichothiodystrophy cells. *Mutation Research* **346:** 107-114.

Sanford KK, Parshad R, Price FM, Tarone RE, Schapiro MB (1993) X-ray-induced chromatid damage in cells from Down syndrome and Alzheimer disease patients in relation to DNA repair and cancer proneness. *Cancer Genetics and Cytogenetics* **70:** 25-30.

Sanford KK, Parshad R, Price FM, Tarone RE, Thompson J, Guerry D (1997) Radiation-induced chromatid breaks and DNA repair in blood lymphocytes of patients with dysplastic nevi and/or cutaneous melanoma. *Journal of Investigative Dermatology* **109:** 546-549.

Sanford KK, Tarone RE, Parshad R, Tucker MA, Greene MH, Jones GM (1987) Hypersensitivity to G2 chromatid radiation damage in familial dysplastic naevus syndrome. *Lancet* **2:** 1111-1116.

Sarasin A (2003) An overview of the mechanisms of mutagenesis and carcinogenesis. *Mutation Research* **544:** 99-106.

Savage JRK (1970) Sites of radiation induced chromosome exchanges *Current Topics in Radiation Research* **6:** 129-194.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S *et al*. (1995a) A single ataxia telangiectasia gene with a product similar to

PI-3 kinase. *Science* **268:** 1749-1753.

Savitsky K, Sfez S, Tagle DA, Ziv Y, Sartiel A, Collins FS, Shiloh Y, Rotman G (1995b) The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. *Human Molecular Genetics* **4:** 2025-2032.

Sax K (1942) The Distribution of X-Ray Induced Chromosomal Aberrations. *Proceedings of the National Academy of Sciences of the United States of America* **28:** 229-233.

Schneiderman MH, Schneiderman GS (1984) G2 cells: progression delay and survival. *Radiation Research* **98:** 389-396.

Schneiderman MH, Schneiderman GS, Rusk CM (1983) A cell kinetic method for the mitotic selection of treated G2 cells. *Cell Tissue Kinetics* **16:** 41-49.

Schwartz JL, Cowan J, Grdina DJ, Weichselbaum RR (1996) Attenuation of G2-phase cell cycle checkpoint control is associated with increased frequencies of unrejoined chromosome breaks in human tumor cells. *Radiation Research* **146:** 139-143.

Scott D (1994) Individual differences in radiation sensitivity: relevance to radiotherapy and cancer predisposition. *Annual Research Report1994, Christie Hospital (NHS) Trust*: 19-22.

Scott D (2000) Chromosomal radiosensitivity, cancer predisposition and response to radiotherapy. *Strahlentherapie Onkologie* **176:** 229-234.

Scott D, Barber JB, Spreadborough AR, Burrill W, Roberts SA (1999) Increased chromosomal radiosensitivity in breast cancer patients: a comparison of two assays. *International Journal of Radiation Biology* **75:** 1-10.

Scott D, Spreadborough A, Levine E, Roberts SA (1994a) Genetic predisposition in breast cancer. *Lancet* **344:** 1444.

Scott D, Spreadborough A, Roberts SA (2003) Less G2 arrest in irradiated cells of breast cancer patients than in female controls: a contribution to their enhanced chromosomal radiosensitivity? *International Journal of Radiation Biology* **79:** 405-411.

Scott D, Spreadborough AR, Jones LA, Roberts SA, Moore CJ (1996) Chromosomal radiosensitivity in G2-phase lymphocytes as an indicator of cancer predisposition. *Radiation Research* **145:** 3-16.

Scott D, Spreadborough AR, Roberts SA (1994b) Radiation-induced G2 delay and spontaneous chromosome aberrations in ataxia-telangiectasia homozygotes and heterozygotes. *International Journal of Radiation Biology* **66:** S157-163.

Scott D, Zampetti-Bosseler F (1982) Cell cycle dependence of mitotic delay in X-irradiated normal and ataxia-telangiectasia fibroblasts. *International Journal of Radiation Biology* and *Related Studies in Physics, Chemistry and Medicine* **42:** 679-683.

Shen SX, Weaver Z, Xu X, Li C, Weinstein M, Chen L, Guan XY, Ried T, Deng CX (1998) A targeted disruption of the murine Brca1 gene causes gamma-irradiation

hypersensitivity and genetic instability. *Oncogene* **17:** 3115-3124.

Shiloh Y, Parshad R, Frydman M, Sanford KK, Portnoi S, Ziv Y, Jones GM (1989) G2 chromosomal radiosensitivity in families with ataxia-telangiectasia. *Human Genetics* **84:** 15-18.

Shiloh Y, Parshad R, Sanford KK, Jones GM (1986) Carrier detection in ataxia-telangiectasia. *Lancet* **1:** 689-690.

Shiloh Y, Tabor E, Becker Y (1983) Abnormal response of ataxia-telangiectasia cells to agents that break the deoxyribose moiety of DNA via a targeted free radical mechanism. *Carcinogenesis* **4:** 1317-1322.

Shiloh Y, Tabor E, Becker Y (1985) In vitro phenotype of ataxia-telangiectasia (AT) fibroblast strains: clues to the nature of the "AT DNA lesion" and the molecular defect in AT. *Kroc Foundation Series* **19:** 111-121.

Sinclair WK (1968) Cyclic x-ray responses in mammalian cells in vitro. *Radiation Research* **33:** 620-643.

Slichenmyer WJ, Nelson WG, Slebos RJ, Kastan MB (1993) Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage. *Cancer Research* **53:** 4164-4168.

Smart V, Curwen GB, Whitehouse CA, Edwards A, Tawn EJ (2003) Chromosomal

radiosensitivity: a study of the chromosomal G2 assay in human blood lymphocytes indicating significant inter-individual variability. *Mutation Research* **528:** 105-110.

Spitz MR, Wu X, Wang Y, Wang LE, Shete S, Amos CI, Guo Z, Lei L, Mohrenweiser H, Wei Q (2001) Modulation of nucleotide excision repair capacity by XPD polymorphisms in lung cancer patients. *Cancer Research* **61:** 1354-1357.

Stern MC, Umbach DM, Lunn RM, Taylor JA (2002) DNA repair gene XRCC3 codon 241 polymorphism, its interaction with smoking and XRCC1 polymorphisms, and bladder cancer risk. *Cancer Epidemiology Biomarkers and Prevention* **11:** 939-943.

Swift M (1976) Malignant disease in heterozygous carriers. *Birth Defects Original Article Series* **12:** 133-144.

Swift M, Morrell D, Cromartie E, Chamberlin AR, Skolnick MH, Bishop DT (1986) The incidence and gene frequency of ataxia-telangiectasia in the United States. *American Journal of Human Genetics* **39:** 573-583.

Swift M, Reitnauer PJ, Morrell D, Chase CL (1987) Breast and other cancers in families with ataxia-telangiectasia. *New England Journal of Medicine* **316:** 1289-1294.

Takai S, Price FM, Sanford KK, Tarone RE, Parshad R (1990) Persistence of chromatid damage after G2 phase X-irradiation in lymphoblastoid cells from Gardner's syndrome. *Carcinogenesis* **11:** 1425-1428.

Taylor AM (2001) Chromosome instability syndromes. *Best Practice & Research Clinical Haematology* **14:** 631-644.

Terasima T, Tolmach LJ (1961) Changes in x-ray sensitivity of HeLa cells during the division cycle. *Nature* **190:** 1210-1211.

Terasima T, Tolmach LJ (1963a) Variations in several responses of HeLa cells to x-irradiation during the division cycle. *Biophys J* **3:** 11-33.

Terasima T, Tolmach LJ (1963b) X-ray sensitivity and DNA synthesis in synchronous populations of HeLa cells. *Science* **140:** 490-492.

Terzoudi GI, Jung T, Hain J, Vrouvas J, Margaritis K, Donta-Bakoyianni C, Makropoulos V, Angelakis P, Pantelias GE (2000) Increased G2 chromosomal radiosensitivity in cancer patients: the role of cdk1/cyclin-B activity level in the mechanisms involved. *International Journal of Radiation Biology* **76:** 607-615.

Thompson LH, Schild D (2001) Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutation Research* **477:** 131-153.

Thompson LH, West MG (2000) XRCC1 keeps DNA from getting stranded. *Mutation Research* **459:** 1-18.

Tice R, Windler G, Bary JM (1978) Effect of cocultivation on sister chromatid exchange frequencies in Bloom's syndrome and normal fibroblast cells. *Nature* **273:** 538-540.

Tuimala J, Szekely G, Wikman H, Jarventaus H, Hirvonen A, Gundy S, Norppa H (2004) Genetic polymorphisms of DNA repair and xenobiotic-metabolizing enzymes: effects on levels of sister chromatid exchanges and chromosomal aberrations. *Mutation Research* **554:** 319-333.

Valerie K, Povirk LF (2003) Regulation and mechanisms of mammalian double-strand break repair. *Oncogene* **22:** 5792-5812.

Vogelstein B, Kinzler KW (1993) The multistep nature of cancer. *Trends in Genetics* **9:** 138-141.

Vral A, Thierens H, Baeyens A, De Ridder L (2002) The micronucleus and G2-phase assays for human blood lymphocytes as biomarkers of individual sensitivity to ionizing radiation: limitations imposed by intraindividual variability. *Radiation Research* **157:** 472-477.

Vral A, Thierens H, Baeyens A, De Ridder L (2004) Chromosomal aberrations and in vitro radiosensitivity: intra-individual versus inter-individual variability. *Toxicology Letters* **149:** 345-352.

Weterings E,van Gent DC (2004) The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair (Amst)* **3:** 1425-1435.

Wikman H, Risch A, Klimek F, Schmezer P, Spiegelhalder B, Dienemann H, Kayser K, Schulz V, Drings P, Bartsch H (2000) hOGG1 polymorphism and loss of heterozygosity

(LOH): significance for lung cancer susceptibility in a caucasian population. *International Journal of Cancer* **88:** 932-937.

Xu B, Kim ST, Lim DS, Kastan MB (2002) Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Molecular and Cellular Biology* **22:** 1049-1059.

Yarosh DB, Pena A, Brown DA (2005) DNA repair gene polymorphisms affect cytotoxicity in the National Cancer Institute human tumour cell line screening panel. *Biomarkers* **10:** 188-202.

Zampetti-Bosseler F, Scott D (1981) Cell death, chromosome damage and mitotic delay in normal human, ataxia telangiectasia and retinoblastoma fibroblasts after x-irradiation. *International Journal of Radiation Biology* and *Related Studies in Physics, Chemistry and Medicine* **39:** 547-558.

Zhou BB, Elledge SJ (2000) The DNA damage response: putting checkpoints in perspective. *Nature* **408:** 433-439.

APPENDICES

APPENDIX A: FIXED CELLS STORAGE LOCATION SHEET

Form: GEN cells-1v1
March 2002
First Issue

Fixed Cells Storage Location Sheet

Box ID ……

1	2	3	4	5	6	7	8	9
10	11	12	13	14	15	16	17	18
19	20	21	22	23	24	25	26	27
28	29	30	31	32	33	34	35	36
37	38	39	40	41	42	43	44	45
46	47	48	49	50	51	52	53	54
55	56	57	58	59	60	61	62	63
64	65	66	67	68	69	70	71	72
73	74	75	76	78	78	79	80	81

APPENDIX B: G_2 RADIOSENSITIVITY SCORE SHEET

Code:- Irradiated ☐ Control ☐ Microscope no.:- Scorer:- Date:-

Slide	No.	CO-ORD	2N	N	chsg	chsb	chtg	chtb	COMMENTS.
	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								
	11								
	12								
	13								
	14								
	15								
	16								
	17								
	18								
	19								
	20								
	21								
	22								
	23								
	24								
	25								
	26								
	27								
	28								
	29								
	30								
	31								
	32								
	33								
	34								
	35								
	36								
	37								
	38								
	39								
	40								
	41								
	42								
	43								
	44								
	45								
	46								
	47								
	48								
	49								
	50								

APPENDIX C: FORMS FOR DANISH FAMILIES

Questionnaire

Indication of Genetic Damage Transmitted to Children of Danish Survivors of Childhood Cancer – A Feasibility Blood Collection Study

Institute of Cancer Epidemiology
Danish Cancer Society
Strandboulevarden 49
DK-2100 Copenhagen Ø

Study no.: _____ Date of interview: _____

1. Basic information

1.1 Sex M F |__|__|

1.2 Age Year |__|__|

2. Cancer in the family

2.1 Has anyone in your nearest biological family had cancer? Yes No Not sure |__|__|__|

(Parents, grandparents, siblings, children, parent's siblings; i.e. aunts and uncles, but not adopted children, stepfamily or family in-laws)

2.2 If yes, please specify:

Family member	Type of cancer/Not sure	ICD-8

3. Smoking habits

	Yes	No
3.1 Are you a current smoker? (1 cigarette per day in 6 months)	|__|	|__|

	Yes	No
3.2 Have you previously been a current smoker? (1 cigarette/day in 6 months)	|__|	|__|

	Number
3.3 How much do/did you smoke in average per day/week/month?	|__|__|
Number of cigarettes	|__|__|
Per day	|__|
Per week	|__|
Per month	|__|

	Age
3.4 Age at start of smoking?	|__|__|

	Age
3.5 Age at quitting, if former smoker?	|__|__|

	Year
3.6 Total years of daily smoking?	|__|__|

4. Medications

4.1 Do you currently use any form of medication?
We ask you about prescription and over-the counter drugs as well as alternative medicine.

Yes No
|__|__|

If yes, please specify the name of the drug(s), duration of use as well as the indication

Drug name	Duration of use in months (m)/years (y)	Indication

4.2 Have you previously received large doses of chemotherapy or similar drugs due to serious illness?

Yes No
|__|__|

Drug name	Duration of use in months (m)/years (y)	Indication

5. Use of Hormones (women only)

Yes No

5.1 Do you use oral contraceptives? |__|__|

If yes, please specify the name of the drug(s) and duration of use

Drug name	Duration of use in months (m)/years (y)

Yes No

5.2 Do you use any other type of hormones, such as estrogens and/or progesterones? |__|__|

If yes, please specify the name of the drug(s), duration of use and type of hormones

Drug name	Duration of use in months (m)/years (y)	Type of hormones*

* Estrogen only, progesterone only, combination pills, others (please specify)

Informed Consent

Indication of Genetic Damage Transmitted to the Children of Danish Survivors of Childhood Cancer – A Feasibility Blood Collection Study

I have read the information brochure, and I hereby confirm that I agree to participate in the study.

Furthermore, I give permission to having my and my child(ren)'s blood drawn.

I understand that participation in the study is entirely voluntary and that I can withdraw my and my child(ren)'s commitment without giving any explanation.

Do you allow your blood sample to be at stored at the Institute of Cancer Biology, Danish Cancer Society, Copenhagen, and to be used in future studies on childhood cancer after renewed approval from the Danish Ethical Committee?

Yes
No

Do you allow your child(ren)'s blood sample to be at stored at the Institute of Cancer Biology, Danish Cancer Society, Copenhagen, and to be used in future studies on childhood cancer after renewed approval from the Danish Ethical Committee?

Yes
No

Date: _____

Name: _____

Signature: _____

Institute of Cancer Epidemiology
Danish Cancer Society
Strandboulevarden 49
DK-2100 Copenhagen, Denmark

Information Pamphlet

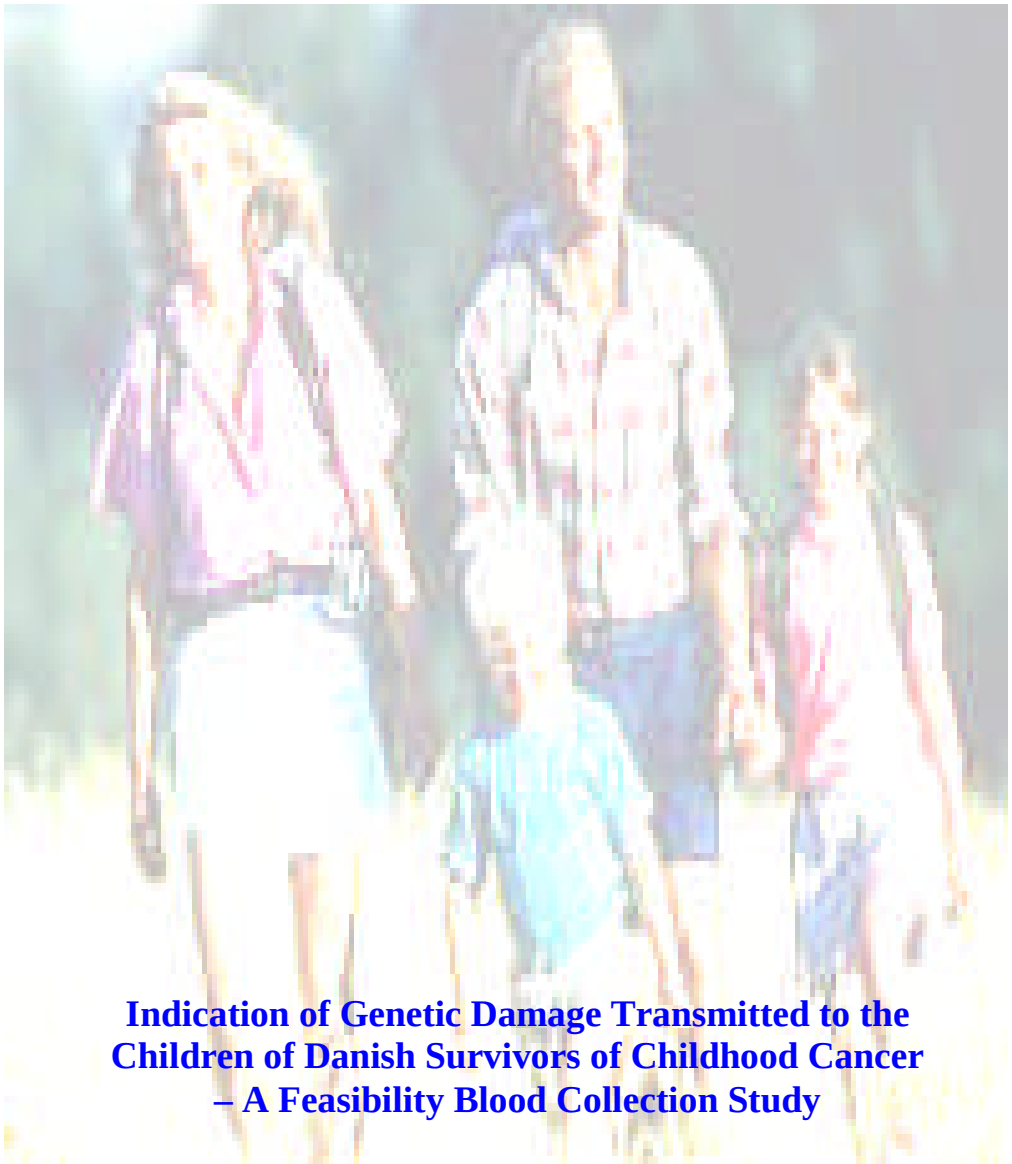
Approved by the Danish Scientific Ethical Committee
If a scientific study of a certain disease or disorder includes human beings the purpose of the study must be to prevent or treat the disease. This is included in the regulations of the Scientific Ethical Committee consisting of both medical doctors and laymen. These regulations are confirmed by law. This scientific investigation is approved by the Scientific Ethical Committee and therefore satisfies the ethical requirements of the committee.

Financial support
The project is funded by the Danish Cancer Society Copenhagen, Denmark and the International Epidemiology Institute in Rockville, Maryland, USA.

Project investigators
Responsible project investigators are Ms. Jeanette Falck Winther, MD and senior researcher and Mr. Jørgen H. Olsen, DMSc and Director, both from the Institute of Cancer Epidemiology, in collaboration with Catherine Rechnitzer, DMSc and in charge of the Late Effect Clinic, Juliane Marie Center, Rigshospitalet, and senior researcher Per Guldberg, Institute of Cancer Biology, Danish Cancer Society. These Danish investigators are part of an international collaboration with researchers from England and the US.

Identity safety
On all blood samples and questionnaires study numbers will replace personal identity numbers. All information gained will be used for tabulations of statistics only where no identification of data at the personal level can be found.

Institute of Cancer Epidemiology
Danish Cancer Society
Strandboulevarden 49
2100 København Ø



Indication of Genetic Damage Transmitted to the Children of Danish Survivors of Childhood Cancer – A Feasibility Blood Collection Study

Background
It is still not evident whether radiotherapy frequently used to treat childhood and adolescent cancer can damage the germ cells (the sperm or the eggs) and whether such alterations at the genetic level, if introduced, are passed onto the next generation.

Fortunately, it is well documented that offspring of patients treated for childhood cancer bear no increased risk for cancer. This also seems to be the case for other serious disorders as e.g. congenital malformations. Nonetheless, it cannot be ruled out that radiotherapy might introduce alterations in the germ cells at the molecular level (mutations) affecting health of the next generation to a minor degree or leading to no clinical observable manifestation at all.

Aim
This is what we would like to investigate – in a laboratory study at first. We hope to be able to enroll 25 families consisting of the parents, one treated for cancer in childhood or adolescence, and all - or at least one - child. The family needs to be resident in Jutland.

By thoroughly investigating and comparing variations in the genomic material of blood cells from the mother, father and children it is possible to find changes, if any, in the child's genes, which are introduced by radiation treatment of one of the parents being the cancer survivor.

How you and your family can contribute
If your reply letter indicates that you and your family wish to participate in our investigation the project coordinator and medical doctor Jeanette Falck Winther will call you to get an appointment for a meeting at the Pediatric Oncology Outpatient Clinic at Skejby hospital (Monday afternoons). When you arrive at the clinic the doctor will help you fill out a short questionnaire. Afterwards you, your partner and child(ren) will be asked to have a blood sample drawn. Reimbursement of transport costs will of course be provided.

Questionnaire
The questionnaire, which will be filled out by you and your partner, includes information on family history of cancer, smoking habits, medication and hormone use (oral contraceptives). With your permission, information of treatment for your childhood cancer will be abstracted from your medical record and included in the study.

Blood sample
Before the meeting, we mail some analgetic plasters for your child(ren) to minimize pain (instruction included). The blood sample from your partner will be used as a control to ensure that any alterations in the genes of your children, if hereditary, are not associated with radiation treatment.

The blood samples will be analyzed at laboratories at Westlakes Research Institute in England. With the permission of you and your partner, the remaining blood will be frozen down and stored in a biobank at the Institute of Cancer Biology situated at the Danish Cancer Society so that the blood can be used for future studies of childhood cancer.

We would like to stress that the result of the analyses based on bloods from you and your family can only reveal a pattern if combined with results of other families participating in the study. Therefore, you and your family will not receive a letter with the result of the analyses based on your own blood samples. Instead you will receive a newsletter in 18 to 24 months when we have finalized the investigation.

Voluntary and confidential
Participation in the investigation is entirely voluntary and you can at any time withdraw your commitment without giving any explanation. All information will be treated confidentially and will not be available for anyone else but the project investigators.

APPENDIX D: GEL LOADING PLAN

Gel Loading Plan

PCR Date:
PCR Operator:
Gel Date:

Gel Number:	**Date:**
Lane:	**Sample:**
1	---
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
16	
17	
18	
19	
20	---

Gel Number:	**Date:**
Lane:	**Sample:**
1	---
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
16	
17	
18	
19	
20	---