Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.
Cellular and molecular mechanisms associated with sensitivity to DNA damaging agents in various human genetic disorders.


Orla Howe

Radiation and Environmental Science Centre.
Dublin Institute of Technology,
Kevin St, Dublin 8,
Ireland

April 2004.
DECLARATION

I declare that this thesis is entirely my own work, except where otherwise stated in the acknowledgements and within. This thesis has not been submitted to this or any other university as an exercise for a degree. I give permission to the library to lend or copy this thesis.

Orla Howe
CONTENTS

Title
Declaration
Table of contents
Acknowledgements
Dedication
Summary
Abbreviations
Communications

CHAPTER 1: Cellular and Molecular Mechanisms of Human Genetic Disorders

1.1 Cancer and Genetic Predisposition
1.2 Cancer Predisposition Genes
1.3 Specific human genetic disorders investigated in this study:
   1.3.1 Breast Cancer
   1.3.2 Prostate Cancer and Benign Prostatic Hyperplasia
   1.3.3 Ataxia-Telangectasia (A-T)
   1.3.4 Ataxia-Oculomotor Apraxia 1 (AOA1)
1.4 Genetic mechanisms of cell cycle regulation
1.5 Mutagenesis as a consequence of DNA damage
1.6 DNA Repair processes in response to DNA damage
1.7 Cell death as a consequence of DNA damage
1.8 Summary of thesis objectives

CHAPTER 3:
The assessment of *in-vitro* responses to radiation potential indicators of radiosensitivity in patients with benign prostatic hyperplasia and prostate cancer

3.1 Introduction 82
3.2 Materials and Methods 89
3.3 Results 98
3.4 Discussion 111

CHAPTER 4:
The relationship between ataxia-telangectasia (ATM) gene carriers and breast cancer predisposition.

4.1 Introduction 121
4.2 Materials and Methods 126
4.3 Results 129
4.4 Discussion 134
CHAPTER 5:
The recessive hereditary ataxias: Characterisation of a complex case through cellular and genetic studies.

5.1 Introduction
5.2 Materials and Methods
5.3 Results
5.4 Discussion

CHAPTER 6:
Ataxia-ocular motor apraxia (AOA1): A study of the sensitivity phenotype in AOA1 “deficient” and “proficient” patient cells.

6.1 Introduction
6.2 Materials and Methods
6.3 Results
6.4 Discussion

CHAPTER 7: GENERAL DISCUSSION

APPENDICES
Appendix 1: Reagents
Appendix 2: Additional methods 230
Appendix 3: Statistical analysis data 234

REFERENCES 244
ACKNOWLEDGEMENTS

I would like to express my gratitude to everybody in both the RESC and QIMR laboratories, who helped me during my PhD professionally and socially. You have all made a huge impact on my life and provided me with many happy memories in Dublin and Brisbane. Unfortunately there are too many of you to thank specifically here, but I am grateful to you all.

First and foremost, I would like to thank Dr. Carmel Mothersill for giving me the opportunity to conduct this work and for her continual advice and guidance throughout. She opened up many doors for me in scientific research such as allowing me to travel to conferences and other laboratories and thus making the years of my PhD a memorable experience.

I would like to thank Prof. Martin Lavin for inviting me to work at his laboratory in QIMR and for providing me with many opportunities in molecular research. My visit to QIMR in Brisbane was a wonderful learning experience in many respects and for this I am very grateful.

A big thanks to Dr. Nuri Gueven who took me under his wing while conducting my molecular research at QIMR. I guess I brought a whole new meaning to the word “patience” for him. I am very grateful for all his help and guidance.

I would like to thank Dr. Fiona Lyng for her all advice and help with my writing up and for the many fun memories.

I would like to thank Dr. Geoff Birrell for all his help and supervision with the dHPLC work. And a big thanks to the rest of the aprataxin group, Dr. Amanda Kijas (for the antibodies), Dr. Olivier Becqueral and Dr. Phil Chen for great team work.
Thanks to Dr. Seymour for those early morning irradiations for the G2 assay.

Thanks to Prof. Peter Daly for his support and providing me with breast cancer samples at the start of my PhD.

Thanks to Dr. Ciaran O’Malley, St. Vincent’s Hospital, Dublin and Dr. Frank Gardiner, Royal Brisbane Hospital, Queensland, for the prostate patient samples.

Thanks to Dr. Natasha Coen and Niamh Kilmurray, two of my bestest friends in the whole world which I also had the pleasure of working with during my PhD. Without them, my sanity would have been questioned at times.

Thanks to James, soon to be Dr. Brown himself. I am completely indebted to him for all his help throughout my PhD. Most of all I thank him for “minding” me in Brisbane and for being a fantastic friend.

Thanks to Aine, my “agony aunt” and the “cell culture guru” for all her help at QIMR and for her much cherished friendship.

Thanks to Tanya, Jess and Kel, for the fun girlie nights out in Brissy to keep me sane.

Thanks to the older R.E.S.C crew, Dr. Michael Kilemade and Dr. Brian Quinn who have gone further afield, to Ed, Sharon and Peter who are not far behind, and to all the rest of the gang in RESC for their camaraderie.
I dedicate this thesis to my parents David and Ruth for their continual encouragement, love and support through all my academic years. And of course to “the Kids” my doggies Gismo and Sooty who made me have many outdoor breaks during my write-up and were always there for a cuddle when it was needed.
SUMMARY

The integrity of the genome is important to cellular and organism homeostasis. It is under constant threat due to intrinsic and extrinsic factors which contribute to a variety of genetic alterations and so genomic integrity may become compromised. However, a complex set of cellular surveillance and repair mechanisms have evolved in cells to respond to this potentially deleterious damage.

The genotype in individuals is also important for the cellular responses to DNA damage and ultimately presents the clinical phenotype in patients. In this study, the sensitivity phenotype of patients with a variety of genetic disorders was assessed to investigate the underlying cellular and molecular mechanisms that occur in response to DNA damage.

The DNA damaging agents were chosen on the basis of their ability to form DNA-single strand and -double strand breaks in cells. Samples from breast cancer patients and prostate patients with benign prostatic hyperplasia and prostate cancer were exposed to ionising radiation (0-0.5Gy) and increased radiosensitivity in these cancer conditions was demonstrated. Ataxia-telangectasia (A-T) is characterised by hypersensitivity to ionising radiation and its correlation to breast cancer is well documented. This association was shown by screening a number of breast cancer DNA samples for specific A-T mutations through denaturing high-performance liquid chromatography (dHPLC). Ataxia oculomotor apraxia (AOA1) which was described as a disease entity mimicking A-T was another condition investigated in this study. A patient encoded ATL2ABR showed clinical similarities to the AOA1 phenotype, and so the expected genotype was APTX which encodes a long and short form of the protein, aprataxin. These cells were exposed to ionising radiation and they showed intermediate radiosensitivity in contrast to A-T cells. APTX was cloned into inducible
vector systems and transfected into ATL2ABR cells. Subsequent radiation sensitivity studies also showed intermediate sensitivity indicating that \textit{APTX} may not be the causative gene. Subsequent dHPLC analysis showed no mutations in \textit{APTX} in ATL2ABR cells. The cells were later shown to be hypersensitive to mitomycin C (MMC) which indicates that a different genotype associated with the underlying molecular and cellular processes were responsible for the clinical phenotype of ATL2ABR.

Further sensitivity studies were carried out on AOA1 patients' cells (L136, L938 and L939). These cells were exposed to various DNA damaging agents with hypersensitivity demonstrated with hydrogen peroxide exposure. Both forms of \textit{APTX} were cloned into suitable vector systems and subsequently transfected into AOA1 cells. These \textit{APTX}-transfected cells showed correction of sensitivity to hydrogen peroxide in contrast to the hypersensitivity observed in the untransfected cells. Furthermore, a mutation 788T$\rightarrow$G (V263G) in the HIT region of both the short and long form of \textit{APTX} was made and also transfected into AOA1 cells. These cells with mutated \textit{APTX} showed hypersensitivity to hydrogen peroxide indicating destabilisation of aprataxin protein. This hypothesis was supported in subsequent mutation studies using enhanced Green fluorescent protein (EGFP) tagged Long form of \textit{APTX}.

The sensitivities of breast and prostate cancer, A-T, ATL2ABR and AOA1 patients elucidate the cellular and molecular mechanisms that occur in response to the DNA damaging agent responsible for the sensitivity. Furthermore, this study shows that the sensitivity genotype is an important factor for the sensitivity phenotype, as sensitivity can be corrected through insertion of the deficient gene as demonstrated in AOA1 patients.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-T</td>
<td>Ataxia-Telangectasia</td>
</tr>
<tr>
<td>AOA</td>
<td>Ataxia-oculomotor apraxia</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>BC</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>IR</td>
<td>Ionising radiation</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methanosulfonate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand breaks</td>
</tr>
<tr>
<td>DSBR</td>
<td>Double-strand break repair</td>
</tr>
<tr>
<td>SSBR</td>
<td>Single-strand break repair</td>
</tr>
<tr>
<td>dHPLC</td>
<td>Dentauring high-performance liquid chromatography</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumor suppressor gene</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDI</td>
<td>Cyclin dependent inhibitor</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>LET</td>
<td>Linear energy transfer</td>
</tr>
</tbody>
</table>
COMMUNICATIONS

G 2 workshop technical report.

Aprataxin, a novel protein that protects against genotoxic stress.

Elevated G2 chromosomal radiosensitivity in Irish breast cancer patients: A comparison with other studies.

PRESENTATIONS

G2 Chromosomal radiosensitivity assay and RNA analysis on Irish breast cancer patients. O.Howe, P.Daly, C.Seymour, D.Byrne and C.Mothersill.


Awarded an "RRS 2001 student travel award".

Explant culture techniques of prostate tissue as a tool for the assessment of low dose radiation effects.

2nd International conference of world council of nuclear workers (WONUC), Dublin Institute of Techology, Dublin, June 27-29. (Poster only)

Discussion of the reliability of the G2 assay observed in the Irish studies to date.
O.Howe, P.Daly, K.O’Malley, C.Seymour, C.Mothersill.

Technical workshop on the G2 assay, St.Andrews University, Scotland, Sept 20-23, 2001

Elevated G2 chromosomal radiosensitivity observed in patients with benign and malignant prostate conditions. O.Howe, K.O’Malley, P.Daly, C.Seymour, C.Mothersill.


Awarded an “RRS 2002 student travel award".

CHAPTER 1

CELLULAR AND MOLECULAR MECHANISMS OF HUMAN GENETIC DISORDERS

1.1 Cancer and genetic predisposition
1.2 Cancer predisposition genes
1.3 Human genetic disorders investigated in this study:
   1.3.1 Prostate cancer and benign prostatic hyperplasia
   1.3.2 Breast cancer
   1.3.3 Ataxia-telangectasia (A-T)
   1.3.4 Ataxia-Oculomotor Apraxia (AOA)
1.4 Genetic mechanisms of cell cycle regulation
1.5 Mutagenesis as a consequence of DNA damage
1.6 DNA repair processes in response to DNA damage
1.7 Cell death mechanisms as a result of DNA damage
1.8 Summary of thesis objectives
1.1 CANCER AND GENETIC PREDISPOSITION:

1.1.1 Cancer and carcinogenesis

Cancer is a process by which normal cells progressively transform to a malignant phenotype from defective cellular processes initiated by genetic alterations within the genome. The genetic basis of cancer can be delineated into two main topics. (A) Genetic predisposition to cancer which is conferred by specific defects that are inherited in families (B) Tumour formation which is genetically determined by alterations in specific tumour cells which accumulate and progress in forming the neoplasia.

Genetic alterations (mutations) occur from either endogenous processes or interaction with exogenous agents. Cancers that arise from these types of mutations are known as spontaneous cancers. In contrast cancers that arise due to their genetic constitution are termed hereditary cancers. Each genetic variant has its own specific molecular origin and may differ from the one leading to the next genetic variation (Arber, 2003), thus contributing to biodiversity between individuals. In some cases, mutations in specific genes confer predisposition to cancer and the cellular and molecular response to these mutations are important in carcinogenesis.

To date more than 200 distinct types of cancers are known (Cancer Research UK, 2003).

Mortality and incidence rates of each cancer are well documented by epidemiologists. The abnormal growth of malignant cells is thought to affect 1 in 4 adults and 1 in 800 children during their lifetimes (Cowel, 2001). Statistical reports show that breast, lung, prostate and colorectal cancer are the most common within the western world (Ries LAG et al, 2003; Cancer Research, UK 2003).
Although each cancer type has its own aetiological factors, the multistep process of carcinogenesis leading to the expressed malignant phenotype is common to all cancers and the cellular mechanism of carcinogenesis has only begun to be elucidated in recent years (Vogelstein et al, 1993). The main feature of carcinogenesis is abnormal cellular proliferation which may occur in part from the molecular events of the cell cycle.

Cellular proliferation is a biological process required by most cells to replenish and repopulate organs and tissues. The cells that have the capacity to do this are known as stem cells and there are approximately $10^{12}$ cell divisions per day in these stem cell compartments in the human body (Bertram, 2003). Cellular proliferation and cell death is controlled by the complex molecular mechanisms of the cell cycle. An alteration in this network of molecular cell cycle mechanisms can cause uncontrolled cellular growth and tumour formation. The molecular mechanisms of cell cycle control will be discussed in more detail later, to understand the carcinogenesis hypothesis to a greater extent.

1.1.2 Cancer predisposition:

As previously described, the acquisition of the malignant phenotype is a progressive and cumulative effect of altered genotypes. These altered genotypes are a consequence of spontaneous effects or inheritance of specific mutated genes which can contribute to cancer predisposition.

Inherited cancers are those which are caused from a genetic defect that is passed to the offspring from a parent which then increases the probability of cancer susceptibility. Galen (A.D. 131 to 203) was the first to describe theories of the origin of cancer.

However, he did not consider cancer with a hereditary component. The earliest report
of cancer within families dates back to 1866 where a French physician Broca, described a family with mostly metastatic breast cancer (Offit, 1998; Gareth and Evans, 1998). Advances in cancer research present numerous studies of genetic predisposition within families. Some of these studies are based on population based statistics (Pharoah, 2003) and the majority of these studies in biological research especially in recent years.

Genetic predisposition to cancer can be categorised into those inherited cancer syndromes (Li Fraumeni syndrome), familial cancer (breast cancer and ovarian cancer), inherited predisposition with evident familial clustering and autosomal recessive syndromes with defective DNA repair (Ataxia-telangectasia). (Kearsley and Lavin, 1994).

The vast majority of mutations in genes that give rise to cancer are not inherited and so genetic susceptibility plays a limited role to neoplastic formation. This was demonstrated by several studies using molecular genetic models on families and twins (Risch, 2001; Lichtenstein, 2000). Studies in breast and ovarian cancer show that between 5-10% are hereditary (Claus et al, 1996) and the remainder are sporadic. Thus, the quest to identify the genes responsible for both hereditary and sporadic cancers is ongoing and rapidly expanding.
1.2 CANCER PREDISPOSITION GENES:

Advances in the scientific field through molecular biology have allowed us to elucidate the specific cancer predisposition genes associated with human genetic disorders. The identification of these cancer predisposition genes is currently an expanding network and many of these genes have been documented (Venkitaraman, 2003; Evans, 1998).

Cancer predisposition genes can be of high or low penetrance. Penetrance is defined as the proportion of individuals with a mutant allele who express the associated trait (Offit, 1998). In other words, the resultant phenotype of an individual will express the physical, biochemical or clinical characteristics of the disease. When discussing the phenotype of an individual, the underlying genotype must be discussed as it is this genetic constitution that forms the phenotypical state.

It is well known that genes from diseases follow the Mendelian mode of inheritance. Experiments carried out by Gregory Mendel in the 1850s described the basic modes of inheritance using peas. He described dominant, recessive and sex-linked inheritance. In his model, individuals have two alleles of a gene. When only one of these altered alleles is required to produce the disease this is known as dominant inheritance and the offspring would be heterozygous for that gene with one altered allele and one normal allele. In contrast recessive inheritance requires two copies of the altered gene (the homozygous state) to express the diseased state. Most syndromes conferring genetic susceptibility follow a dominant pattern of inheritance. Some of these syndromes and their genetic predisposition genes are well established such as breast cancer (Wooster and Wever, 2003; Gayther et al, 1998) and prostate cancer (Verhage and Kiemeney, 2003) and other syndromes and their causative genes reviewed by D.G. Evans (1998).
In recent years syndromes which follow a recessive mode of inheritance and their associated cancer predisposition genes have been described. Syndromes such as ataxia-telangectasia (Savitsky et al, 1995), and other autosomal recessive syndromes such as Fanconi anaemia and Blooms syndrome are examples of these. Furthermore, it has been shown that certain recessive susceptibility genes can interact with certain dominant susceptibility genes. Thus, they are versatile, in that they are individually responsible for one or more disease. An example of this was shown by Howlet et al (2002), where a mutation in the breast cancer susceptibility genes BRCA2 may cause Fanconi anemia. Another example of this is the increased interest in the association of the ATM gene and its predisposition to breast cancer (Angele et al, 2003; Angele and Hall, 2000; Swift et al, 1987).

This suggests the importance of recessive predisposition genes as well as the dominant ones in human genetic diseases. Therefore, molecular biologists now endeavour to identify low penetrance cancer predisposition genes as well as high penetrance genes. The use of mouse models is one such approach and is reviewed by de Koning et al (2003). The well established cell and molecular biological techniques are used extensively for this purpose.

Predisposition genes are characterised not only by their structural and aetiological features but also by their function on a molecular basis. There are three main classes of cancer susceptibility genes which have been studied extensively in recent years. These are known as the oncogenes, tumor suppressor genes and DNA repair genes. Oncogenes, tumour suppressor genes and DNA repair genes all function in response a genetic alteration in the genome if it arises spontaneously or it is inherited. Oncogenes and tumor suppressor genes are also involved in the normal molecular mechanisms of the cell cycle to assist cellular proliferation or cell death. Oncogenes
are genes which are activated by a mutation, whereas tumour suppressor genes are those which are inactivated by a mutation (Bertram, 2001). DNA repair genes are those which are activated in their repair pathway in response to specific DNA damage. Researchers have concentrated mainly on tumour suppressor genes in cancer research due to their highly penetrant phenotype; however interest has increased in mutations in oncogenes and DNA repair genes (Gareth and Evans, 1998). These genes and their implications in cancer and cancer predisposition will be discussed. However, elucidation of the underlying genetic mechanisms will be better delineated in the later sections on cell cycle, DNA damage and DNA repair processes.

1.2.1. Oncogenes

Oncogenes are defined as viral or cellular genes that are involved in cell growth and proliferation and when they are over-expressed, amplified or mutated, they can induce neoplastic transformation or cancer (Offit, 1998). Proto-oncogenes are the inactive form of oncogenes. They are activated by an increased amount of the gene product or an abnormal gene product being formed by a mutation in the gene. More than 70 different oncogenes have been identified to date (Marx et al, 1994). Oncogenes code for oncoproteins, which are classified according to their biologic function. These include protein kinases, growth factors such as, signal transducers and nuclear transcription factors, and associations of oncogenes with various cancers have been made.

The ras genes, which code for a family of signal transduction molecules, was the first oncogene to be discovered in human cancer cells. It represents the most widely activated oncogene found in human cancers with estimations of up to 90% in colon cancer and 30% in lung cancer (Bertram, 2001).
Growth factor oncogenes such Erb-B (which is a membrane receptor for its ligand heregulin) was suggested to be over-expressed in 30% of breast carcinomas and is associated with worst clinical outcome (Bertram, 2001).

Other examples of oncogenes implicated in various cancers are the c-abl gene of which its protein product has elevated tyrosine kinase activity in chronic myelogenous leukaemia, the c-myc family which are transcription factor oncogenes and are found in many tumors as they drive cellular proliferation and the Bcl-2 family associated with apoptosis of regulating cell numbers of different cell types. Several studies on the association of oncogenes with the various cancers have been carried out such as those on breast cancer (deJong et al, 2002), and prostate cancer (reviewed by Karan et al (2003) and Prendergast and Walther (1995)).

1.2.2 Tumour Suppressor Genes (TSG):

Tumour suppressor genes are defined as genes that code for proteins that suppress tumor formation by acting as negative growth regulators (Hoffee, 1998). Tumor suppressor genes play an important role in cell cycle progression. They function by activating or repressing transcription of specific genes within the cell cycle.

Tumour suppressor genes differ from oncogenes in their mutagenic activation. Mutation in a single allele of an oncogene would cause a gain of function and neoplastic formation. However both alleles of the TSG must be inactivated for loss of function to occur. Mutations in a single allele of a TSG would be silent and result in inheritance of the mutation. However, this can be detrimental in individuals predisposed to cancer (Bertram, 2001).

Well known examples of TSGs are P53 which has described as the commonest genetic change found in human cancers (Lane D, 1994) and PTEN in Cowdens
syndrome which confers genetic susceptibility to other cancers. Many other highly penetrant autosomal dominant genes conferring susceptibility and which are tumour suppressor genes are known. Examples of these are BRCA1 (Hall et al, 1990; Miki et al, 1994) and BRCA2 (Wooster et al, 1995) in breast cancer, and RB in retinoblastoma (Lane et al, 1993; Picksley et al, 1994) which join an expanding list (Gareth and Evans, 1998). Genetic analysis of the functions of the tumour suppressor genes with inherited predisposition to cancer has also been reviewed by Presneau et al (2003). P53 tumour suppressor gene is further described here because of its important role in cell cycle events and the implications of the genetic defects in P53 for cancer predisposition.

**P53 tumour suppressor gene:**

P53 has been referred to as the guardian of the genome (Lane D.P, 1994). When DNA damage occurs, it can trigger cell cycle arrest to allow repair processes to occur or trigger apoptosis to prevent tumour formation. Therefore its main function is to monitor genome integrity and function directly in DNA replication, repair and recombination in response to DNA damage (Morris, 2002). Activation of P53 occurs in response to DNA damage where it becomes post-translationally modified by phosphorylation and acetylation events. An associated protein known as Mdm2 (Picksley et al, 1993) is released leading to the activation of P53 which acts as a transcription factor to induce genes downstream causing cell cycle arrest (mainly G1 and G2 phase), repair (mainly DNA excision repair) or apoptosis. P53 is recognised as the most commonly mutated gene in human malignancies (Lane, 1994) and has been estimated in over 70% of human cancers (Bertram, 2001).
1.2.3. DNA Repair genes:

The third type of cancer susceptibility genes are those involved in DNA repair. As their name suggests, these genes are involved in the repair response to DNA damage from carcinogens and intrinsic cytotoxic compounds. They monitor the chromosomes to correct damaged nucleotide residues. XRCC protein variants (Price et al, 1997) and DNA repair Ligase proteins are some examples of the predisposition genes that join a growing list (Wood et al, 2001). These are discussed in more detail in section 1.6 on DNA repair processes.
1.3 SPECIFIC HUMAN GENETIC DISORDERS RELEVANT TO THIS STUDY

1.3.1 Breast cancer

Breast cancer is the most common malignancy in women in the western world affecting approximately one in fourteen in Ireland (Irish Cancer Society, 2004), one in ten in the UK and one in nine in the US (Angele and Hall, 2000). Breast cancer incidence is due to various risk factors such as age, endogenous factors (such as inherited predisposition, hormonal imbalance etc) and exogenous factors (such as diet, mutagen exposure etc) (Gayther et al, 1998).

Breast cancer cases are described as sporadic or hereditary. Sporadic breast cancers arise spontaneously in nature however hereditary cancers arise due to inheritance of an altered genotype. It has been estimated that about 5-10% of breast cancers are inherited while the remaining 90-95% are sporadic (Rosen, 2003). Various high-penetrance and low-penetrance predisposition genes have been identified in both hereditary and sporadic breast cancers. Linkage analysis studies have identified two important highly-penetrant breast cancer predisposition genes, BRCA1 which was mapped to chromosome 17q21 (Hall et al, 1990) and was later cloned and identified by Miki et al (1994). BRCA2 was then mapped to chromosome 13q12-13 (Wooster et al, 1994). Linkage analysis studies have also indicated a third putative breast cancer predisposition gene (BRCA3) on the short arm of chromosome 8 in the region of 8p12-22 (Kerangueven et al, 1995), however, no somatic or germline mutations have been detected in the putative gene in breast cancer cases (de Jong et al, 2002).

Functional studies have been carried out on these genes to identify proteins that they interact with directly or indirectly. The roles that BRCA1 and BRCA2 play in
maintaining genomic integrity through interactions with cell cycle and DNA repair has recently been determined, and stems from studies demonstrating the activity of the BRCT domain at the carboxyl terminus of BRCA1 protein product. It was hypothesised that BRCA1 is a cell-cycle dependent nuclear protein that is regulated by cyclin dependent kinases (DePotter et al., 1998). BRCA has been shown to interact with proteins such as those involved in transcription, DNA repair, cell cycle regulatory proteins and tumour suppressor proteins as reviewed by Rosen (2003). Studies on BRCA2 showed that it was structurally different from BRCA1 in that it consisted of eight BRC repeats (Bork et al., 1996). In several studies these BRC repeats were shown to interact with RAD51 (Sharan et al., 1997; Scully et al., 1997; Wong et al. 1997) and other similar proteins that interact with BRCA1 which were mentioned previously, suggesting its role in transcriptional regulation, cell cycle and DNA repair. Thus it was suggested that both proteins may function in the same or overlapping pathways (Sharan et al., 1997).

Mutations of BRCA1 and BRCA2 were thought to account for 65-80% of hereditary breast cancer and < 5% of all breast cancer (Gayther et al., 1998; Easton et al., 1995), therefore implicating additional genes involved in breast cancer predisposition. Highly penetrant genes such as those responsible for rare genetic syndromes have been shown to be associated with the risk of developing breast cancer. These include the TP53 gene (Li-Fraumeni syndrome) which has been found in breast cancer (Harris and Holstein, 1993) of which inherited mutations are thought to account for less than 1% and somatic mutations accounting for 19-57% (deJong et al., 2002). Mutations in genes such as PTEN/MMAC/TEP gene (Cowdens syndrome) and STK11/LKB1 gene (Peutz-Jegher’s syndrome) have also been implicated in the increased risk of predisposition to breast cancer (Arver et al., 2000) by possible interactions with
BRCA1 and 2 or other proteins associated with the activation and inactivation of the genes in cell cycle events.

Low penetrance genes have also been identified and associated with breast cancer. ATM from the autosomal recessive human disorder Ataxia-Telangiectasia (A-T) is one of these genes and was first reported by Swift et al (1987). The relative risk of breast cancer in A-T heterozygotes varies from approximately 4% (Easton D.F., 1994) to 12.7% (Stankovic et al., 1998) and this risk factor was reviewed by Angele and Hall (2000).

As previously suggested, other breast cancer predisposition genes of low penetrance may be those involved in cell cycle and DNA repair processes, of which some may interact directly with non-mutated BRCA1 and BRCA2 (mainly sporadic cases) or indirectly such as a downstream signalling protein.

These breast cancer predisposition genes (with the exception of BRCA1 and BRCA2) along with other recently identified genes (high penetrance and low penetrance) were reviewed by deJong (2002). In this review he used Pubmed database searches from 1980-2000 and reviewed the associated susceptibility. However with an increase in interest in this field of research, the list is rapidly growing.

These breast cancer predisposition genes and their molecular mechanisms will be elaborated upon further in the proceeding chapter, specifically on Breast cancer.

1.3.2 Prostate cancer and benign prostatic hyperplasia (BPH)

The two main types of prostate diseases in males are prostate cancer and benign prostatic hyperplasia (BPH). Both have similar characteristics in that they occur from uncontrolled growth, they are associated with androgen dependence and the incidence increases with increasing age. However, BPH is the non-malignant condition in the
prostate which differs from the malignant form in prostate cancers associated with tumorigenesis. Furthermore, BPH is not a premalignant lesion, or a precursor of prostate cancer. Each condition will be described here in brief but elaborated further in later chapters.

1.3.2.1 Prostate Cancer:

Prostate cancer has become a major health problem among males the Western world. It is the most common diagnosed malignancy and the second leading cause of cancer death. Approximately 100,000 men are newly diagnosed each year in the EU (Hamdy, 2001) and approximately 200,000 men were diagnosed in the US in 2001 (Karan et al, 2003). Prostate cancer incidence is affected by various risk factors. The most prominent risk factor is age. Prostate cancer is more prevalent in men over 50. Brawley et al (1998) showed that approximately 75% of prostate cancers are diagnosed in men between 50-70 years. It has been demonstrated that incidence rates vary between different ethnic groups with the largest incidence rate found in African-American males and the lowest incidence in men from Eastern countries such as Japan and China (Muir et al, 1987). However, additional exogenous risk factors such as diet and lifestyle and endogenous risk factors such as hormonal imbalance and genetic predisposition could contribute to this difference in incidence between the ethnic groups.

Genetic predisposition to prostate cancer was first evident from a study carried out by Woolf et al in 1960 utilising the Utah cancer registry. They determined the risk of death from prostate cancer to be threefold higher in first-degree male relatives of men who had died from the disease than in men with no such relatives (Kainu and Issacs,
Epidemiological studies are consistent with this hypothesis such as the recent study by Stanford and Ostrander (2001).

However, although there is evidence of genetic predisposition to cancer, the quest to identify these genes has been more difficult than in other cancers such as breast and colon cancer, which suggests that prostate cancer is probably due to multiple genes of moderate or low penetrance rather than several highly penetrant genes. However, segregation analysis studies which determine the likely mode of inheritance and penetrance of prostate cancer susceptibility genes have been carried out by various groups (Gronberg et al, 1997; Schiad et al, 1998; Verhage et al, 2001). These studies are consistent in supporting the presence of at least one highly-penetrant autosomal dominant prostate cancer susceptibility gene.

Subsequent linkage analysis studies were carried out to search for these highly-penetrant prostate cancer predisposition genes. The first putative locus for a prostate cancer gene found was HPC1 by Smith et al (1996) at chromosome 1q24-25. PCAP was the second putative locus reported by Berthon et al (1998) at chromosome 1q42.2-43. The identification of several other putative loci for prostate cancer genes soon followed. These were HPCX at Xq27-q28 (Xu et al, 1998), CAPB at 1q36 (Gibbs et al, 1999), HPC20 at 20q13 (Berry et al, 2000), 8p22-23 (Xu et al, 2001) and HPC2/ELAC at 17p11 (Tavtigian et al, 2001).

Several candidate loci for medium to low-penetrance prostate cancer predisposition have been proposed such as those involved in androgen metabolism such as HSD17B2 mapped to chromosome 16q24.1-q24.2, and SRD5A2 mapped to chromosome 2p23-22 (Verhage and Kiemeney, 2003; Singh et al, 2000).

High-penetrance genes from other diseases have been implicated with prostate cancer development such as BRCA1 and BRCA2 breast cancer genes. It was proposed that
BRCA1 carriers have a 6% lifetime risk factor, whereas BRCA2 carriers have between 6-12% risk of developing prostate cancer (Ford et al, 1998)

1.3.2.2 Benign Prostatic Hyperplasia (BPH):

BPH is the non-malignant, uncontrolled growth of cells in the prostate. As mentioned previously, incidence of BPH increases in men over the age of 50. The causes of BPH are unknown however hormonal imbalances have been implicated in its development. The male hormone testosterone can be converted to a testosterone derivative dihydrotestosterone (DHT) by the enzyme 5-α-reductase in some tissues and estradiol in others. DHT is more potent than testosterone and can accumulate and stimulate cellular proliferation causing hyperplasia (Wright et al, 1999).

1.3.3 Ataxia-Telangectasia

Ataxia-Telangectasia (A-T) is an autosomally recessive condition which is characterised by progressive cerebellar ataxia and ocultaneous telangectasia, immunodeficiency, severe radiation sensitivity, and cancer predisposition (Sedgwick and Boder, 1991; Lavin and Shiloh, 1997; Gatti, 1998). It was first described by Syllaba and Henner in 1926 as a pleiotrophic phenotype with involvement in multiple organs (Iannuzzi et al, 2002).

Ataxia-Telangectasia arises due to the inheritance of mutations in the ATM (mutated) gene. ATM was mapped to chromosome 11q22-q23 by Savitsky et al (1995). They demonstrated that ATM spans 150 kilobases of genomic DNA, contains 66 exons and produces a 13-kb mRNA transcript which encodes a protein of 3,056 amino acids. This protein is 370kDa in size and was shown to contain a domain related to that in the phosphatidylinositol 3-kinases (P13-kinases) superfamily (Lavin et al, 1995). It
plays an important function in DNA double strand break repair by initiating a
signalling cascade within the cell cycle and functioning as a cell cycle checkpoint
protein. It has been shown to interact with cell cycle proteins such as BRCA1, ABL,
TP53 and protein complexes such as the MRN (MRE11/RAD50/Nibrin) complex

Mutations in ATM have been linked to cancer predisposition. Homozygous and
compound heterozygous A-T patients exhibit severe clinical phenotypes. For
example, Morrell et al (1986) estimated that 38% of A-T homozygotes patients
develop leukaemia and lymphoma more frequently (Sommer et al, 2003). However,
heterozygous carriers of ATM mutations are mainly asymptomatic. Approximately
1% of the general population are A-T heterozygous carriers (Swift et al, 1986).
Several studies suggest that heterozygous carriers of ATM mutations have an
increased risk to cancer susceptibility. Breast cancer susceptibility in women has
been related to A-T heterozygous carriers. Various epidemiological studies showed
an increased risk in breast cancer among family members of A-T patients. These
studies were pooled and analysed by Easton (1994). He estimated that 3.8% of breast
cancer cases would be attributable to A-T. More recent studies have shown similar

1.3.4 Ataxia-Oculomotor Apraxia 1 (AOA1)

Oculomotor apraxia was first described by Cogan in 1953 as a syndrome presenting
jerky head movements in patients. However, Aicardi et al (1988) associated these
clinical features in 14 patients with a specific disease described as Ataxia with
oculomotor apraxia. It was described as a disease entity mimicking A-T, which has
similar neurological features but it does not have the extraneurological featureless of A-
T such as immunodeficiency and sensitivity to ionizing radiation. The neurological features of Ataxia-oculomotor apraxia are ataxia, oculomotor apraxia and choreoathetosis. It is a condition which occurs due to an autosomal recessive inheritance pattern.

Linkage analysis studies have shown that AOA is classified into two subtypes according to the genetic locus. AOA1 which was mapped to chromosome to 9p13 in Portuguese and Japanese families (Moreira et al, 2001a) and AOA2 which was mapped to chromosome 9q34 in Japanese and Pakistani families (Nemeth, et al, 2000; Bomont et al, 2000). These AOA2 families presented a clinical phenotype similar to AOA1 but they had elevated alpha-fetoprotein levels.

Mutations in the causative gene, APTX are responsible for AOA1 which was mapped to chromosome 9p13 (Moreira et al, 2001b; Date et al, 2001). The gene responsible for AOA2 maps to chromosome 9q34 and has recently been identified and was called senataxin (Moreira et al, 2004). Several families with Oculomotor apraxia do not show linkage to either of these loci which suggests the possibility that other loci may be involved in each AOA (reviewed by Coutinho et al, 2002).

The causative gene of AOA1, APTX was found to encode a histidine-triad (HIT) protein which was called aprataxin (Moreira et al, 2001b; Date et al, 2001). They presented two major mRNA species encoding a long and a short form of aprataxin as a result of alternative splicing on exon 3. The long form encodes a 342-amino acid protein. It consists of PANT domain (PNKP-aprataxin amino terminal) which is a forkhead associated domain (FHA), a middle domain of a HIT motif, and a carboxy-terminal domain containing a zinc-finger motif. The short form encodes a 168 amino acid protein but does not have the FHA domain.
Studies are currently being carried out on proteins that interact with aprataxin. It was hypothesised that aprataxin may be involved in single-strand break repair. Recent studies show that aprataxin interacts with XRCC1 which forms a multiprotein complex in the single-stranded break repair process (Sano et al, 2004; Gueven et al, 2004).

AOA1 and AOA2 are among a group of autosomal recessive ataxias which are rare neurodegenerative diseases. The most common autosomal recessive ataxias in Europe are Friedreich's ataxia and ataxia-telangectasia. Less common forms are spinocerebellar ataxia, AOA and others reviewed by DiDonato et al (2001). The relative frequency of AOA has been estimated at 5% (LeBer et al, 2003) and 7.5% (Barbot et al, 2001; Moreira et al, 2001) among these studies.
1.4 GENETIC MECHANISMS OF CELL CYCLE REGULATION:

To understand the mechanism of carcinogenesis, it is important to fully understand the cell cycle and its regulatory elements, as it is defects within these that cause uncontrolled cellular proliferation and cancer formation.

The cell cycle is a set of biological events which a cell requires to transmit genetic information from one generation to another. Although mitosis was first described in 1875, it was not until 1951 through experiments carried out by Alma Howard and Steve Pelc on the roots of vicia fabia seedlings, that the concept of the cell cycle was demonstrated (Baserga, 1999). They described the cycle as having four distinct phases. S Phase (DNA synthesis), mitosis, and two intermediate phases, G1 and G2 which are between S phase and mitosis. Subsequent experiments showed the movement of cells from one phase to another within the cell cycle in a variety of cells using tritiated thymidine (\(^{3}\)H-Tdr) and by 1962, Stanley Cohen described growth factors as the driving factors of the cell cycle (Baserga, 1999). Since then, our knowledge on the cell cycle and the underlying genetic mechanisms has greatly increased.

The use of yeast cells (\textit{saccharomyces cerevisiae}) has been a powerful tool for studying the cell cycle at a molecular biological level, and has provided the framework for our understanding of the mammalian cell cycle. This system allowed the identification of cyclins and their respective roles in the cell cycle and cell cycle progression (Puri et al, 1999).

Progression through the cell cycle involves various cell cycle transitions. These are defined as unidirectional changes in which a cell shifts its activity to perform a new
set of processes (Puri et al, 1999). Cell cycle transitions are dependent on cyclin-dependent kinase regulation.

1.4.1 Cyclin-dependent kinase regulation:

Cell cycle transitions depend on the activity of cyclin-dependent kinases (CDKs). CDKs are activated by binding to their cyclin partners and act as positive regulators, whereas cyclin dependent kinase inhibitors (CKIs) act as negative regulators because they act as breaks to stop cell cycle progression in response to regulatory signals (Park; Lee, 2003). It is regulation of these CDKs that is responsible for critical cell cycle events such as cell growth (G1), DNA replication (S) and cell division (G2/M).

1. Cyclin-dependent kinases (CDKs):

Cyclin-dependent kinases are a family of serine-threonine protein kinases. They are activated by binding to a cyclin subunit and forming a CDK-cyclin complex, at specific stages of the cell cycle and they activate downstream proteins by phosphorylation. To date, nine CDKs have been identified. Five of these are active during the cell cycle. CDK4, CDK6 and CDK2 are active during G1 phase, CDK2 during S phase and CDK1 during M phase. CDK7 works in combination with cyclin H (Vermeulen et al, 2003). In contrast, the remaining four CDKs have not been shown to have any crucial roles in normal cell cycle control (Rickert et al, 1996).

Monomeric CDKs are completely inactive in the absence of cyclins and their protein levels remain stable. However, the protein concentrations of cyclins vary throughout the cell cycle and it these levels that cause subsequent CDK activation (Vermeulen et al, 2003). All CDKs share a sequence related to the canonical EGVPSTAIRISLLKE motif in domain III initially demonstrated in yeast (Puri et al, 1999). This region is
important for the binding of CDKs to cyclins in conjunction with another sequence
that includes a threonine residue in domain VII (Pines 1996). This process is
facilitated by another cyclin/CDk complex known as CAK which is composed of
cyclin H, CDK 7 and a third protein known as MAT1 (Fisher et al, 1995). It was
proposed that most cyclin/CDK complexes are possible substrates for CAK (Puri et al,
1999).

The various CDKs and their associated cyclins required for activation at specific
phases of the cell cycle are shown in figure 1.1.

2. Cyclin-dependent kinase inhibitors (CDKIs):
The function of cyclin-dependent kinase inhibitors is to inhibit the activity of CDKs
after they function, so that their activity is regulated in cell cycle progression. CDIs
have been defined in terms of two classes. One class is known as the cip-kip family
(or the universal CDI). This family includes proteins such as P21, P27 and P57 and
they function by inhibiting the G1 kinases (CDK3, CDK4 and CDK6). The second
class of CKIs are known as the INK4 family (or the specific CDI). It is composed of
P15, P16, P18 and P19, and they function by inhibiting the activity of CDK4 and
CDK6 (Puri et al, 1999). These proteins function when they are expressed at high
levels. Thus, over-expression of these genes can cause cell cycle arrest.
Figure 1.1: The stages of the cell cycle and the associated CDK/Cyclin complexes.

1.4.2 Cell cycle checkpoint regulation:

The biochemical pathways that alter cell cycle transitions are known as cell cycle checkpoints. These checkpoints have been described into two classes; the checkpoints that respond to DNA damage, and the checkpoints that monitor DNA synthesis pathways (reviewed by Nyberg et al, 2003). Both constitute a network of signal transduction mechanisms which regulates cell cycle progression. The main cell cycle checkpoints are the G1/S phase, G2/M phase and the S-phase checkpoint. The main function of the G1 checkpoint is to block the activity of the CDK2-Cyclin E complex. Similarly, the main function of the G2 checkpoint is to block the activity of the CDK1-Cyclin B complex. The functions of the S-phase checkpoint are still being hypothesised, but processing and stabilisation of the stalled replication forks along with homologus recombination appear to be the main functions and are the focus of current research. The mechanisms which are associated with each important checkpoint will be explained in more detail.

1.4.3. The molecular mechanisms of cell cycle regulation in response to DNA damage:

Genomic integrity is under threat from DNA damaging agents both intrinsic and extrinsic. These agents target DNA and form lesions. It is thought that these different types of lesions are converted into single-strand DNA (ssDNA) or double-strand breaks (DSBs). Various proteins within the cell are activated in response to these types of DNA damage. It is proposed that ssDNA activates a checkpoint pathway containing the ATR kinase protein and DSBs activate a checkpoint pathway containing the ATM protein (Yang et al, 2004; Nyberg et al, 2003). Both of these proteins are vital for detecting DNA damage and initiating the activation of other
important proteins such as CHK1 and CHK2 (at the G1 and G2 checkpoints) which interfere with normal control of the cell cycle by inactivating several cyclin-dependent kinases. In contrast, a separate set of proteins and mechanisms have been elucidated for the S-phase checkpoint pathway.

1. Cell cycle checkpoint proteins associated with DNA damage:
The proteins involved in checkpoint control of DNA damage and replication have been described and divided into three main groups (Nyberg et al, 2003; Iliakis et al, 2003).

(A) The Sensor proteins: These proteins function to recognise and signal DNA damage and initiate subsequent processes in response to the damage. Examples of sensor proteins are the MRN (Mre11-Rad50-Nbs1) complex in response to double-strand breaks, BRCA 1 and the replication proteins. (B) The transducer proteins: These are mainly protein kinases which respond to the signals from sensor proteins by activating other proteins downstream through phosphorylation events. Examples of these transducer proteins are ATM and ATR, phosphoinositide 3-kinases and Chk1 and Chk2, serine-threonine kinases. (C) The effector proteins: These are proteins activated by the transducer kinase proteins further downstream, in response to the initial DNA damage signal, and can halt cell cycle progression. Figure 1.2 illustrates these types of proteins in the cellular processes that occur in response to DNA damage.
2. G 1 – S phase and G 2 – M phase checkpoints:

(A) The role of ATR:

The ATR-ssDNA checkpoint pathway involves interaction of a set of proteins which may bind directly or indirectly to the transducer protein and then subsequently amplifies the damage signal to cause cell cycle arrest. It was suggested that the Rad17-RFC pentamer may bind to the ssDNA and also allow the 9-1-1 (Rad9-Rad1-hus1) heterotrimer to bind to the DNA for activation of ATR upon DNA damage (Zou and Elledge, 2002). These may recruit substrates such as BRCA 1 which initiate the phosphorylation of the ATR-ATRIP heterodimer. It was also postulated that the ATR-ATRIP heterodimer may bind to ssDNA independently (Nyberg et al, 2003).

Activation of the ATR-ATRIP complex plays a role in the G1 checkpoint by activating P53 by phosphorylation at serine 15. This results in P21 activation and subsequently a block in CDK2/cyclinE complex which prevents progression of G1 to S phase.

Activation of the ATR-ATRIP complex similarly plays a role in the G2 checkpoint. CHK1 is activated by phosphorylation at serine 317/345 (Guo et al, 2000). This subsequently causes phosphorylation at serine 549 on the WEE1/14-3-3 complex which blocks CDC25C phosphatase activity, and also directly phosphorylates CDC25C at serine 216 which promotes the binding of 14-3-3 proteins and also blocking its function in the cell cycle progression of G2 phase to Mitosis. Figure 1.3 and 1.4 illustrate the cellular pathways of ATR at the G2/M and G1/S checkpoints.
(B) The role of ATM:

The ATM-DSB pathway also involves a series of proteins upon activation. Figure 1.3 and 1.4 illustrate these pathways in cell cycle checkpoint control. It has been proposed that ATM is activated by intramolecular autophosphorylation on serine 1981 (Bakkenist and Kasten, 2003) in response to DSBs. It then associates with NBS1 (Carson et al, 2003; Buscemi et al, 2001; Zhao et al, 2000, Gatei et al, 2000) a component of the MRN complex (Mre11-Rad50-NBS1) which acts as a damage sensor triggering a cascade of events involving the phosphorylation of proteins at cell cycle checkpoints. In the G1 checkpoint, activated ATM has been shown to activate proteins such as CHK2 (Matsuoka et al, 2000) by phosphorylation at threonine 68 which then phosphorylates P53 on serine 20 and/or CDC25A at serine 123 which in the latter can cause degradation or inactivation of the CDC25A complex. It can activate MDM by phosphorylation on serine 395 which then inhibits its binding to P53 promoting stabilisation. ATM can also directly activate P53 by phosphorylation at serine 15. The activation of P53 causes induction of P21 which then inhibits the CDK2-Cyclin E complex and inhibiting the progression of the G1 phase to the replication (S) phase.

Other targets of ATM in the G1 checkpoint includes novel proteins such as MDC-1 (Stewart et al, 2003), E2F1 (Slansky et al, 1996), and SMC1 (Kim et al, 2002) but their contribution to the G1 checkpoint is unclear (Iliakis et al, 2003). In the G2 checkpoint, activated ATM directly phosphorylates BRCA 1, however the consequences of this phosphorylation is unknown (Nyberg et al, 2003). Activated ATM causes phosphorylation of CHK 2 at threonine 68 which causes phosphorylation of CDC25C/14-3-3 complex at serine 216 which in turn inhibits the action of CDC2-Cyclin B complex and therefore halting the progression of the G2 phase to M phase.
See figure 1.3 and figure 1.4 for a diagrammatic representation of the G1 and G2 checkpoints and the functions of ATM and ATR in response to DNA damage.

(C) The role of CHK1 and CHK2:

CHK1 and CHK2 are regulatory proteins for both of the checkpoints G1 – S and G2 – M. They function by phosphorylating and inhibiting the Cdc25 homologues required for activation of both the CDK2-CyclinE in the G1 checkpoint and CDK1-CyclinB in the G2 checkpoint as described above and they are activated by ATM and ATR phosphorylation. CHK1 preferentially by ATR and CHK2 preferentially by ATM. In the G1 checkpoint, CHK2 activates CDC25A by phosphorylating it at serine 123. As a result CDK2-Cyclin E and CDK4-Cyclin D are inactivated and furthermore the phosphorylation of CDC25A leads to subsequent degradation as described by Mailand et al (2000). CHK2 also helps stabilise P53 by uncoupling it from Mdm2 ubiquitin ligase, from phosphorylation at serine 20 (Bartek and Lucas, 2001). In the G2 checkpoint the CDC25C complex can be phosphorylated by both CHK1 and CHK2 which maintains it inactive in the cytosol bound to 14-3-3.

CDC25C is also constitutively phosphorylated by C-TAK1 and the function of this is to prevent premature mitosis (Peng et al 1998). Thus CDC25C remains inactive in S-phase because of this. When the cell progresses from S-phase to G2, C-TAK1 must be switched off so that the cell can rely on CHK1 and CHK2 to function in the presence of DNA damage, so that entry from G2 to mitosis is blocked (Eastman, 2004).

3. S-phase (replication) checkpoint control:

DNA damage along with other cellular phenomena such as topological stress, tightly bound protein complexes and nucleotide pools causes a halt in the replication forks
due to S-phase checkpoint control. It was proposed that stalled replicated forks can generate ssDNA and DSB and that homologous recombination is used to repair the damaged replication forks. The proteins associated with checkpoint activity of the replication forks are the DNA helicases Sgs1 and Srs1 found in yeast studies (Barbour and Xiao). It was hypothesised that both of these enzymes are involved in pathways of processing the DNA damage such as the homologous recombination events through activation of proteins such as Mec1 and rad53. They proposed that Srs2 can shuttle cells into the RAD6-dependent pathway when homologous templates are not available and also prevent inappropriate recombination. Sgs1 which helps stabilise the stalled replication forks and prevent inappropriate recombination (Barbour and Xiao). A novel protein encoded by the MGS1 gene has been shown to be involved in homologous recombination (Hishida et al, 2001), and may also be involved in the replication checkpoint control.
**Figure 1.2:**

Diagrammatic representation of the cellular processes that occur in response to DNA damage and determine the fate of the cell.
Figure 1.3: Nyberg et al, 2003

Schematic representation of the G1 checkpoint which shows the detection of DNA damage by ATM and ATR transducer proteins and the signal transduction response from these proteins to others subsequently inactivating the CDK2-Cyclin E complex and preventing the progression of the G1 phase to DNA synthesis (S-phase). Grey arrows indicate loss of function of the protein from inactivation caused by phosphorylation. Sites of phosphorylation are indicated by the amino acid sites labelled on the proteins.
Figure 1.4: Nyberg et al, 2003

Schematic representation of the G2 checkpoint which shows the DNA damage signal transduction response from transducer proteins ATM and ATR to CHK1 and CHK2 and downstream signalled proteins, which blocks CDC2-Cyclin B activity by phosphorylating CDC25C which is complexed to 14-3-3. This causes an arrest in the cell cycle from the G2 phase into mitosis (M-phase). Grey arrows denote loss of function of the protein. Labelled amino acids on proteins denote phosphorylation sites.
1.5 MUTAGENESIS AS A CONSEQUENCE OF DNA DAMAGE:

1.5.1. Mutation and the potential mechanisms for their production

The integrity of the genome is one of the prerequisites of life as it is important to cellular and organism homeostasis. The fidelity of the genome is under constant threat from both intrinsic and extrinsic DNA damaging factors and so it is compromised. It has become accepted that this loss of stability in the genome is one of the most important prerequisites of cancer. (Weismuller et al, 2002; Colleu-Durel et al, 2001).

A permanent change in the nucleotide sequence of a DNA molecule is termed a mutation. (Hoffee, 1998). It is this heritable alteration in the genetic material that is the underlying cause of all human genetic diseases. However, genetic alterations from endogenous processes and exogenous factors can also be contributory factors. There are different types of mutation known. The two main classes are those which are described as gross mutations (Montelone, 1998) as they are detected as cytological abnormalities such as chromosomal gaps and breaks. These chromosomal anomalies which are either spontaneous or induced have been studied for over a century from basic cytogenetic techniques to the more recently studied fluorescent in-situ hybridisation techniques (Natarajan, 2002).

The second main class of mutations are described as point alterations, as they cannot be detected visually as they often involve a single base change in the DNA molecule. This class of mutations can be further subdivided into various types.

(A) Single-base pair substitutions: These are also known as point mutations, where one base pair such as a purine-purine or pyrimidine-pyrimidine is replaced by the other, resulting in the change of a codon, which is the coding sequence of the gene ultimately altering the structure of the gene product.
(B) Insertions or deletions: These are also known as frameshift mutations. They are caused by the insertion or deletion of one or two base pairs within the coding sequence of the gene and altering the reading frame of the gene.

Mutations can occur spontaneously from intrinsic cellular processes, such as errors that are potentially produced during and after DNA replication of undamaged template DNA, mutagenic nucleotide substrates and endogenous DNA lesions (reviewed by Maki, 2002). Spontaneous mutations are those which occur within the cell environment and can be distinguished from induced mutations which involve environmental mutagens to initiate the production of the mutation. It is also possible that spontaneous mutations can be a product of naturally occurring environmental mutagens. The mutagenic activities that occur spontaneously within the cell environment will be discussed here. Mutations that arise from environmental DNA damaging mutagens will be discussed at a later stage.

Analysis of spontaneous mutations in various organisms, have demonstrated that they have a strong bias in their site distribution and in the types of alteration they cause in the genome sequence (Drake, 1991). The content of the genomic sequence such as DNA topology and high order structures in the DNA are responsible for this so called 'hotspot' bias of mutagenic site distribution (Jain and Ranganathan, 2003). Other attributory factors such as the directionality of the replication fork in the transcription process have been speculated (Yoshiyama and Maki, 2003).

The molecular mechanisms of spontaneous mutagenesis have been elucidated through analysis of genes and their products through various organisms such as the bacterium *Escherichia coli* (Kamiya et al, 2003, Miller, 1996), yeast organisms *saccharomyces cerevisiae* (Schmuckli-Maurer et al, 2003), plant organisms such as the grape *phylloxera* (Downie, 2003) and also mammalian cell culture systems (Melnikova et al,
2004; Tateishi et al, 2003). Furthermore, it has been shown that the mechanisms are evolutionarily well conserved between the various organisms (Arber, 2003; Radicella et al, 1997; Slupska et al, 1996).

It was hypothesised from studies on UV and chemical mutagenesis (Friedberg et al, 1995) that every mutation is derived from pre-mutagenic damage of DNA, which can cause a mutagenic intermediate through the normal replication process (misreplication) or from a polymerase that is involved in the synthesis of translesion DNA (TLS). (Maki et al, 2002). This hypothesis has been branded the mutation theory. This pre-mutation is converted to a mutation following errors that are made during the replication process. Three main types of replication errors have been described; a single-base mispair leading to a base substitution, a single-base bulge leading to a single-base frameshift and a multiple-base mismatch leading to a sequence substitution (Maki, 2002).

Mutagenic nucleotides are also involved in the formation of spontaneous mutations. The nucleotides of DNA are subjected to spontaneous structural alteration. This process is called tautomerization. The base guanine for example is capable of existing in two biochemical forms known as the keto and enol forms in which they both can convert between one form and the other. These forms are known as tautomers (Gorb et al, 2002; Blas et al, 2004). The enol form is the destructive form of the two structural isoforms and can occur by shifting a proton or some electrons (Montelone, 1998).

Mutagenic substrate nucleotides can also arise from hydrolytic decay involving depurination, deamination, DNA methylation and DNA bases and strand damage by reactive oxygen species. These types of spontaneous damage were estimated to occur at a frequency of 1-3 x 10^-4 per cell per day (Carr and Hoekstra, 1995).
Attempts have been made to estimate spontaneous mutation rates through mathematical models. By estimating the mutation rate in cancer cells, it can increase the knowledge of the disease and its progression. A study carried out by Natarajan et al (2003) on a human colorectal cell line describes one of these mathematical models. However, the mutation formation system that occurs either cytologically or in response to DNA damage is so diverse and complex that mathematical models to calculate spontaneous mutation rates have limited parameters and are therefore not ultimately a reliable system.

1.5.2. Mutagens

A mutagen can be described as an external agent that can alter the physical composition of DNA and subsequently alter the genetic code. They interact with bases in the genomic sequence and interfere with the normal DNA replication processes which result in the formation of mutations. It was postulated that most mutagens begin their action at the DNA level by forming carcinogen-DNA adducts (Dipple, 1995). These DNA adducts result from the covalent binding of a mutagen or part of a mutagen to a nucleotide base in the structure of DNA (Goldman and Shields, 2003). Several types of exogenous mutagens are known and they are now recognised as environmental carcinogens. However, with a growing realisation that cancers could be linked to mutagen exposure, the list of potential mutagens has been rapidly expanding (Bertram, 2001). Furthermore, it has been estimated that approximately 70% of cancer in Western populations can be attributed to diet and lifestyle (Bertram, 2001).

Mutagens can be classified into chemical and physical mutagenic agents. They both similarly target the nucleus of the cell or more specifically DNA, to form the various
types of mutations previously described, by interacting with the genomic structure and triggering a cascade of cellular mechanisms in response to the exogenous damage.

1.5.2.1 Chemical mutagenic agents:
The earliest example of chemical mutagen carcinogenesis was reported in 1761 by Dr. John who noted unusual tumours in the noses of heavy snuff users. In 1775 a similar observation was made by a surgeon Sir Percivall Pott who observed tumour induction in workers (chimney sweeps) exposed to coal tar. These observations amongst others, led to the hypothesis that cancer results from the action of chemicals on the body. Since then, numerous chemical mutagens have been identified and it is an enormous and expanding field in the pursuit of revealing other chemical mutagens to which individuals are exposed, on a daily basis from their environment.

Chemical mutagens can be base analogues in which they are structurally similar to purines and pyrimidines and during the replication process they may be incorporated into the structure of DNA (e.g. Bromouracil used in scientific research can alter the backbone of DNA by incorporating Uracil). Other chemical mutagens are those which alter the structure or base properties of DNA (e.g. nitric acid formed from digestion of nitrates which act as preservatives in foodstuffs can form bulky DNA adducts and covalently bind to DNA forming strand breaks etc.). Yet another group of chemical mutagens are those which interact with the structure of DNA such as intercalating agents (e.g. ethidium bromide) and other agents which cause inter and intrastrand crosslinks with DNA (e.g. mitomycin C) and DNA breaks (e.g. hydrogen peroxide).

Chemical mutagens can be found in most aspects of our daily life and are often unknown and unavoidable. One such aspect is diet and dietary behaviours. It is now
known that some food mutagens can increase cancer risk contributing to cancer along the route of exposure (oral cavity, gastrointestinal tract) and in distant organs to the route of exposure (e.g. liver). Some of those foodstuffs identified to date include aflatoxin B1, which can be present in mould-contaminated corn and animal feed. It has been established that aflatoxin plays a role in liver carcinogenesis (Groopman and Kensler, 1999).

Other suspected food mutagens are N-nitrosamines which are present in cured meat and fish, and have been linked to oesophageal and other gastrointestinal cancers (Mirvish, 1995), heterocyclic amines which are formed by high temperature cooking of psoralens in meat and vegetables and associated mainly with breast (Zheng et al, 1998) and colon cancer, and some other cancers (Augustsson et al, 1999) and finally polycyclic aromatic hydrocarbons (PAH). PAHs are found in foods such as charred and broiled meat and they have been shown to be related to breast cancer (Rundle et al, 2000) and many other cancers such as lung, oropharyngeal, gastrointestinal and genitourinary tracts (Goldman and Shields, 2003).

Genetic predisposition of these mutagens with the various cancers have been linked to endogenous enzymes which are induced to metabolically activate and detoxify the body of these chemical compounds. The main activating enzymes with genetic polymorphisms responsible for cancer predisposition from these potential food mutagens are the cytochrome P 450 (CYP) enzymes and the N-actelytransferase (NAT) enzymes. The association of these enzymes with the various cancers have been documented (Hunter et al, 1997; Hengstler et al, 1998; Shimada and Fujii-Kuriyama, 2004).

As already mentioned polycyclic aromatic hydrocarbons which are potential food mutagen, are associated with a variety of cancers. However, this can be attributed to
the fact that they are not only found in foodstuffs. They are widespread in the ambient environment due to fossil fuel combustion for energy production, transportation, energy and industry. They can be also found in tobacco smoke and ingested actively or passively in the environment (Rundle, 2000). In urban and industrial areas, PAH levels may be increased, causing atmospheric pollution. Studies carried out in both urban and industrial sites in South America associated mutagenic activity with the presence of PAHs (Vargas, 2003).

Other industrial exposure to chemical mutagens may be occupational. N-nitrosamines were discovered accidentally in workers with liver damage from the exposure of a chemical solvent. It is now known that N-nitrosamines are present in a large number of consumer items such as beer, tobacco smoke and cosmetics (Hecht, 1997). Potential occupational chemical exposure depends on the individual’s occupation. Individuals may be exposed to various heavy metals such as cobalt which is used for the production of alloys and hard metal (De Boeck, 2003), cadmium exposure which occurs from industrial processes (Coen et al, 2001) and nickel exposure which is related to nickel refining, electroplating and welding (Kasprzak et al, 2003). Overall, chemical mutagen exposure can occur depending on an individual’s lifestyle. Occupation and diet (as discussed previously), alcohol consumption (Riedel et al, 2003), smoking (Morabia, 2000) and other factors (e.g. the use of fragrances (Bickers et al, 2003)) have been studied as potential chemical mutagens and thus cancer risk factors.

1.5.2.2 Physical mutagenic agents:

From early discoveries by Roentgen and Becquerel in the late 1800’s, radiation was the first mutagenic agent known. It is the physical transfer of energy from one place
to another by means of particles or waves. In recent years, radiation has been more widespread in its applications for energy production for industrial, military and medical purposes (Morgan, 2003). There are two basic types of radiation. Ionizing radiation which is that which has enough kinetic energy so that during an interaction with an atom, it can remove tightly bound electrons from their orbits, causing the atom to become charged or ionized (e.g. Gamma rays and X-rays). Ionizing radiation can be further described according to the linear energy transfer. LET-Linear energy transfer is the term used to describe the density of ionization in particle tracks. LET is the average energy in KeV given up by a charged particle traversing a distance of 1 μm. Alpha particles would be described as high LET ionizing radiation and gamma rays and X-rays as low LET ionizing radiation. On the other end of the electromagnetic spectrum is the second type of radiation. This is non-ionizing radiation which is radiation that does not ionize the atoms around it (e.g. UV and microwaves).

When ionizing radiation is exposed to matter, it may activate diverse physical and chemical effects including heat generation, atomic displacements, electronic excitation of atoms and molecules, breaking of chemical bonds and molecules, depending on the radiation type, conditions and target (Grossweiner, 2004). Particle track mechanisms of ionizing radiation along with radiation dosimetry are important features for radiation scientists to understand the subsequent effects that occur from ionizing radiation. Radiation forms instantaneous highly structured tracks of atomic ionizations and excitations along the paths. There are various mechanisms in which gamma ray photons are attenuated as they traverse a medium. These mechanisms are known as a simple scatter, photoelectric effect, Compton scatter and pair production depending on the energy of the photons (Grossweiner, 2004).
It is through these various particle track pathways, that exposure of cells to radiation results in the deposition of energy in the cell. The biological effects of ionizing radiation have been well studied for many years and the central dogma for radiobiology has been that the nucleus, or more specifically the DNA, is the target for biological effects of radiation exposure. Studies were also carried out to elucidate the importance of radiation exposure in the cytoplasm of the cell, however, it was concluded that DNA is the critical molecule after exposure showing the cytoplasm to be less sensitive to alpha particles than DNA (Zirkle et al, 1953; Munro TR, 1970).

Ionizing radiation produces a variety of lesions in DNA, including single-strand breaks (SSBs), double-strand breaks (DSBs) and base damage. These lesions are consequences of direct or targeted radiation exposure, as the cell is directly ‘hit’ by an ionizing radiation track, targets the DNA and gives rise to lethal or phenotypic genetic alterations and potentially induces many types of cancer. This is the paradigm of radiation carcinogenesis. However in recent years this has been challenged with phenomena that occur as a result of indirect consequences from radiation exposure. Thus, the cells are not directly ‘hit’ by an ionizing radiation track so molecular disruptions occur at distances from the radiations direct interaction site.

1. Radiation-induced phenomena

In recent radiobiological studies there appears to be a paradigm shift in the field with increasing interests in the indirect, non-targeted and delayed effects of radiation. These effects include radiation-induced genomic instability, death-inducing and bystander effects, adaptive responses, clastogenic factors and transgenerational effects (reviewed by Morgan, 2003).
A. Radiation-induced genomic instability:

Radiation-induced genomic instability (RIGI) can be defined as high levels of non-clonal mutations in the progeny of apparent healthy cells that have survived radiation exposure (Mothersill et al, 2004). RIGI encompasses a multitude of diverse biological endpoints including, chromosomal aberrations, karyotypic abnormalities, sister chromatid exchanges, micronuclei formation, gene mutation and amplification, delayed reproductive death/lethal mutation and cellular transformation (Seymour et al, 1986; Kadhim et al, 1992; 1995; Mothersill et al 1996; 1997a; Little et al, 1997; Lorimore et al, 1998). These effects are regarded as delayed effects resulting from transmissible genomic instability which is induced at frequencies greater than naturally spontaneous mutations (Wright 1998)

These radiation-induced effects can be propagated over time producing a destabilised genotype in the progeny. This was demonstrated in cell biological techniques in vivo (Watson et al, 1997; Kadhim et al, 1992) and in vitro (Ullrich, 1999; Seymour et al, 1986). It is postulated that this propagation of radiation damage from parent cells to further progeny cells may be from a number of processes including reactive oxygen species (Karanjawala et al, 2002; Limoli et al, 1998; Clutton et al, 1996), gap junction communication (Azzam et al, 2001) and also through interaction with some of the previously mentioned indirect effects, such as death inducing factors shown by Nagar et al (2003).

The relevant feature of the endpoints of RIGI concerning biologic risk is that each of these endpoints can persist for multiple generations after exposure, which has led to the proposal that genomic instability may be important in the carcinogenesis cascade.
B. Radiation-induced bystander effects:

Radiation-induced bystander effects (RIBE) has also been widely reviewed by radiation biologists (Mothersill and Seymour, 2003; Lorimore and Wright, 2003; Morgan, 2003b; Goldberg 2003; Mothersill and Seymour, 2001) and its implications for cancer (Mothersill and Seymour, 2004).

This phenomenon has been described as effects that occur in cells that have not themselves been irradiated but have received a signal from an unirradiated cell. RIBE has been described in the literature as far back as 1954, when Parsons et al reported that clastogenic factors that cause damage to chromosomal structures could be detected in the blood of unirradiated patients.

Interest in this phenomenon increased, with the experiments performed by Nagasawa and Little (1992), where less than 1% of the cell nuclei were traversed with low doses of alpha particles, but 30-50% of increased frequency of sister chromatid exchanges in the total cell population were calculated. However, there was speculation about the cells on an individual basis. It was unknown which cells were ‘hit’ and which were not. Thus, the bystander hypothesis was strengthened with the introduction of a single-particle microbeam device (Prise et al, 1998; Randers-Pehrson et al, 2001; Zhou at al, 2003) which functioned by allowing individual charged particles to be targeted into individual cells within a population (Michael, 1999).

Studies on low doses of high LET (Lehnert et al, 1997) and low LET radiation (Mothersill and Seymour, 1997) showed that extracellular factors are involved in the bystander effect. Studies carried out by Emerit et al (1985; 1994; 1995) showed the presence of radiation induced clastogenic factors in radiotherapy patients. Their data suggested that the factor was a low molecular weight molecule (1000-10,000 Da) which was possibly a protein, and that lipid peroxidation and oxidative stress
pathways were involved (Mothersill and Seymour, 2001). Other studies on the characteristics of these extracellular factors proposed that it was a heat labile factor, can survive freeze-thawing, and could be inhibited by superoxide dismutase (Lehnert, Goodwin and Deshpande, 1997). Furthermore it was found that these factors can be produced independently of gap-junction mediated transfer from cell to cell (Mothersill and Seymour, 1998) but was dependent on the numbers of cells present at the time of irradiation (Mothersill and Seymour, 1997) and appeared be prominent at low doses of low LET (Seymour and Mothersill, 2000) and low doses of high LET alpha particles (Little, 2002). Although several factors with potential characteristics of the bystander factor(s) have been documented, the bystander factor(s) responsible for the bystander effect has not yet been identified (Mothersill and Seymour, 2003).

Some of the biological endpoints of radiation-induced bystander effects show chromosomal rearrangments, micronuclei, cell transformation, gene mutations and cell killing (reviewed by Morgan, 2003).

**Interaction of indirect radiation induced phenomena:**

A relationship between the bystander effect and genomic instability has been reported from experiments carried out in vivo (Lorimore et al, 2001) and in vitro (Seymour and Mothersill, 1998). This instability was found to be frequent and nonclonal radiation-induced mutations were observed in daughter cells that were seen in the parent cell (Mothersill and Seymour, 2003).

Radiation induced genomic instability and bystander effects are the indirect non-targeted radiation induced phenomena which have been of increasing interest to radiation biologists and underlie a shift in the conventional radiation carcinogenesis paradigm. However, the other non-targeted radiation induced phenomena mentioned
previously such as the death inducing effect (demonstrated by Nagar (2003)), clastogenic factors and transgenerational effects which have been reviewed by Morgan (2003), are also important manifestations of indirect effects and may interact to form a radiation induced instability phenotype and which may play a role in the multistep carcinogenic process. Other manifestations of delayed effects include transgenerational effects, such as those documented from studies carried out on the Chernobyl incident in 1986. This accident resulted in an unprecedented release of a wide spectrum of radionucleotides in regions of the former USSR. Mutation rates (measured by minisatellite loci) in human populations of Belarus, Ukraine and Russia were estimated in children of parents exposed to the radiation leakage showing transgenerational effects in mainly the father. Mortality and morbidity rates among the offspring were also recorded, showing the radiation induced instability phenotype to have a lethal effect (Dubrova et al, 1998; 2002).

2. Radiation-induced Oxidative Damage:

Damage to DNA by oxidative stress is observed in the bases and sugar-phosphates in the structure of DNA, as well as single-strand breaks and double strand breaks. Single-strand breaks and double strand breaks can be caused from direct ionizing radiation. However, indirect damage can be caused by radicals generated from radiation and result in base damage. Most of these indirect effects occur by free radicals in water, since this makes up 70-80% of mammalian cells. It is the fate of free radicals which are designated \( R^* \) produced in the target, that are important (Horsman and Overgaard, 1997). These radicals react with other molecules to form reactive oxygen species (ROS). The most important of these are \( O_2^* \) (superoxide radical), \( OH^* \) (hydroxyl radical) and \( H_2O_2 \) (hydrogen peroxide).
More than 20 different types of base damage as a result of oxidative stress have been identified. The most common oxidative damage to purines is 7,8-dihydro-8-oxoguanine (8-oxodGuo) where the conformation is able to mispair with adenine causing a transversion of G to T (Martinez, 2003). The most common oxidative damage to the pyrimidines is the formation of thymine glycol (Tg) (Slupphaug et al, 2003). These mechanisms of base damage as a result of oxidative stress were reviewed by Dizdaroglu (1999) and the mechanisms of repair of this oxidative damage were reviewed by Slupphaug et al (2003).

3. Radiation phenotypes: (Radiosensitivity and Radioresistance)

Patients vary in their normal tissue response to a course of radiation exposure.

Human tumor cell lines in culture show a wide range of radiosensitivity and radioresistance, and the intrinsic radiosensitivity of a tumour is an important determinant of a patient’s response to radiotherapy.

Various predictive assays to determine the intrinsic radiosensitivity of a tumour have been demonstrated. One of the most commonly used methods for measuring radiosensitivity in cells is the clonogenic assay (or the colony forming assay) of Puck and Marcus (1973; 1956). Studies have correlated clonogenic survival with clinical outcome using tumor specimens (Bjork-Eriksson et al, 2000). Other studies using clonogenic survival on cell lines derived from a single tumour type to correlate it with radiosensitivity have been less successful (Eastham, 2001). However, this method is too time-consuming for routine clinical application and this has led to the development of assays that measure radiosensitivity manifested by DNA and chromosome damage. Such assays include the G 2 chromosomal radiosensitivity assay (Baria et al, 2001; Papworth et al, 2001), the micronucleus assay (Widel et al,
1999) and the comet assay (Marple, et al., 1998). Some of these methods amongst others to correlate in vitro radiosensitivity and clinical outcome were assessed by Bentzen and Hendry (1999) following a workshop in 1998 on the variability in the radiosensitivity of normal cells and tissues.

In recent years, there has been increasing interest in investigating molecular markers of radiosensitivity for understanding the complex mechanisms underlying a radiosensitive or resistant phenotype. Studies manipulating the expression of certain genes in various pathways have led to a better understanding of the underlying basis of genetic radiation sensitivity. Some of the manipulation strategies described have been those involved in damage recognition, signal transduction, cell cycle checkpoints, transcription factors and repair proteins. These manipulation strategies and their influence on radiosensitivity have all been discussed by Begg and Vens (2001).

DNA double strand breaks are thought to be one of the principal potentially lethal lesions induced by ionizing radiation and repair of these is crucial if the cell is to survive (Begg and Vens, 2001). DSBs may arise through staggered breaks in the phosphodiester backbone of DNA on opposite strands or by the formation of complex damage which is converted to a DSB (reviewed by Rothkamm and Lobrich, 2002). The primary mechanism for repairing DSBs is non-homologous end-joining. This involves a DNA-dependent protein kinase (DNA-PK) comprising the DNA catalytic subunit, Ku70/Ku86 heterodimer, XRCC 4 and DNA ligase IV. These factors have been shown in radiosensitive mutant rodent models (Mori et al., 2001) and the relationship of radiosensitivity and double strand breaks has also been shown in mammalian cell culture systems using, rodent and human cell lines which were repair competent cells and cells with reduced repair capacities (Dikomey et al., 1998).
1.6 DNA REPAIR PROCESSES IN RESPONSE TO DNA DAMAGE

As discussed in the previous sections, the integrity of the genome is under constant threat from endogenous and exogenous factors, contributing to a variety of genetic alterations. These include single strand breaks, double strand breaks, chemically modified bases, abasic sites, bulky adducts, strand cross-links and base-pairing mismatches. Most genomic damage can contribute to mutagenesis, carcinogenesis or cellular lethality.

A complex set of cellular surveillance and repair mechanisms have evolved in cells to respond to this potentially deleterious damage.

DNA repair pathways can be divided into two major groups. The first of these is the repair of DNA double strand breaks, which occurs in mammalian cells by homologous recombination (HR) or non-homologous end joining (NHEJ). The second major group of DNA repair processes are those which act on DNA base damage. This group can be further subdivided into nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER) and reversal of base damage.

The genetic mechanisms which drive these DNA repair processes employ a variety of DNA repair genes of which their protein products are involved in the signalling and regulation of the various DNA repair pathways. These DNA repair genes and pathways were first elucidated from studies on yeast (*Saccharomyces cerevisiae*) in lower eukaryotes and studies on mutant rodent cell lines and patients with defective DNA repair systems in higher eukaryotic systems (reviewed by Hanawalt et al, 1989). Many of these proteins have been conserved in evolution but the nomenclature does not reflect this (Carr and Hoekstra, 1995).

More recent lists of these DNA repair genes have been compiled on the basis of searches in the human genome sequence, however the functions of some of these
genes has yet to be elucidated (Wood et al, 2001). These genes have been grouped according to the known DNA repair processes that they are involved in. Each of these various DNA repair pathways will be discussed separately and will include the genes which are associated with that pathway.

1. DNA strand break repair processes:

Single-strand breaks and double strand breaks can arise from damage to structural components of the genome through endogenous and exogenous sources. If single-strand breaks are not repaired they can be converted to double-strand breaks during the DNA replication process. Double strand breaks are more complex and can result in genomic instability and cell death. Thus double strand break repair mechanisms are also more complex.

1.1 Single-strand break repair (SSBR):

Single strand breaks can be formed directly as mentioned above or indirectly via the enzymatic excision at a site of base damage by AP endonuclease (APE1) during base excision repair (BER) (Caldecott, 2001). The process of single-strand break repair has been divided into four basic steps; Damage binding, end-processing, gap filling and ligation.

Indirect single-strand break repair was created as a result of APE1. This possesses a 5'-deoxyribose phosphate (dRP). Polymerase β inserts a nucleotide (2-15 nucleotides in other cases) into the gap at the damaged site, and removes the 5'-dRP by lyase activity after this gap filling step. The final step in this indirect single-strand break repair process is ligation to restore the DNA structural backbone (Caldecott, 2003).
Direct single-strand break repair was suggested to rapidly bind to poly (ADP-ribose) polymerase-1 (PARP-1) (Shall and De Murcia, 2000) in the damage binding step. In the processing step of the 3' and 5' ends, PARP-1 recruits the molecular scaffold protein XRCC1 (Masson et al, 1998; EI-Khamisy et al, 2003) which stimulates and interacts with polynucleotide kinase (PNK) activity (Whitehouse et al, 2001). PNK has a 3'-phosphatase and a 5'-kinase activity which restores breaks at the 3'-phosphate end and the 5'-hydroxyl end (Jilani et al, 1999, Kairmi-busheri, 1998). XRCC1 and PNK are associated in multiprotein complexes with polymerases (polβ) and ligase III (Whitehouse et al, 2001; Nash et al, 1997) which are thought to facilitate the final single-strand break repair processes of gap filling and final DNA ligation.

1.2 Double-strand break repair (DSBR)

Double-strand breaks repair in mammalian cells occur from two main pathways; Homologous recombination (HR) and non-homologous end joining (NHEJ). The pathway that is used depends on the phase of the cell cycle. NHEJ mainly occurs in G0/G1 whereas HR occurs during the late S and G2 phases (Johnson and Jasin, 2000). Rad52 and KU protein complexes are central to HR and NHEJ respectively. It is thought that these compete for binding to the damaged DNA ends and therefore determining which repair process is used to repair the damaged DNA. HR and NHEJ are error-free and error prone pathways respectively (Christmann et al, 2003).

**Homologous recombination** is not only important for the repair of other complex forms of DNA damage such as interstrand crosslinks or DNA adducts in close proximity on opposite strands. However, DNA double-strand breaks are considered the most lethal.
It is initiated by a nucleolytic resection of the DSB in the 5’-3’ direction by the MRN complex (MRE11/RAD50/NBS1). The single stranded DNA is then bound to a protein complex of a ring structure which is formed by RAD52 proteins. Rad 52 binds to RPA-ssDNA complex to form rad52-RPA-ssDNA nucleoprotein co-complex. This complex facilitates the association of RAD52 onto ssDNA. Following this Rad52 recruits Rad51 to promote strand exchange of an intact sister chromatid or homologous chromosome (Henning and Sturzbecher, 2003). Rad54 is recruited by rad51. Rad54 was shown to have dsDNA-dependent ATPase activity which is used for unwinding the dsDNA (Petukhova et al, 1999). The assembly of the rad51 nucleoprotein is facilitated by various rad51 paralogues (rad51B, rad51C, rad51D, XRCC2 and XRCC3) (Henning and Sturzbecher, 2003; Christmann et al, 2003). After DNA synthesis, ligation and branch migration, the resulting structure is resolved.

Non-homologous end joining is initiated with the binding of the proteins Ku70 and Ku80 heterodimers to the damaged DNA ends. The heterodimer is then bound to DNA-PK which is activated by interaction with a single-strand DNA at the site of the double strand break. The DNA-PK complex interacts with XRCC4 which is bound to DNA ligase IV. This stable complex binds to the ends of the DNA molecules and ligates together duplex molecules with complementary ends which are non-ligatable (Lee et al, 2003), (Reviewed by Christmann et al, 2003; Critchlow and Jackson, 1998; Kanaar et al, 1998). PNK has recently been shown to be involved in repairing non-ligatable 5’-OH ends. The 5’-OH moiety to a 5’-phosphate is catalysed by PNK. It was hypothesised that this mechanism is inter-dependent on NHEJ. Thus, NHEJ
cannot occur without phosphorylation and phosphorylation requires the proper assembly of the NHEJ end-binding proteins (Chappell et al, 2002) to occur.

2. **Repair of Base damage:**

Repair of base damage in the genome can be carried out by excision repair pathways. These are processes which repair chemical damage, mismatches or small loops in DNA, in which the single-stranded section containing the aberrant structure is removed and the resultant gap is filled by DNA replication that is templated from the complementary strand (Cline and Hanawalt, 2003). The excision pathways are base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). DNA base damage reversal by specialised enzymes is another repair pathway of bases in the genome.

2.1 **Base excision repair (BER)**

BER is the replacement of DNA bases that are altered by small chemical modifications through the excision of one nucleotide (short-patch) or 2-13 nucleotides (long-patch) (Cline and Hanawalt, 2003). It is the main repair strategy for repairing DNA damage mainly derived from endogenous sources. Spontaneous hydrolytic depurination and deamination of residues, damage from reactive oxygen species, DNA adducts formed as a result of exposure to reactive metabolites and coenzymes are the types of endogenous base damage which require base excision repair (Lindahl et al, 1997).

The BER process is initiated by a group of enzymes known as DNA glycosylases. These enzymes are involved in the recognition of the base damage, base removal and incision step to BER. These enzymes bind selectively to the damaged base and
hydrolyse the N-glycosidic bond to release the altered base from the genome. 11 mammalian glycosylases have been found to date and their functions known in BER (Christmann et al, 2003). Some glycosylases remove the modified base and leave the AP site, whereas others cleave the AP site giving rise to a single strand break. The AP site is incised in the phosphodiester bond by AP-endonucleases which result in 5' - deoxyribose-5-phosphate (5'-dRP). The next step of BER is nucleotide insertion and the pathway continues by inserting a complementary nucleotide by a polymerase (polβ) into the AP site. The decision between short and long patch repair is the next step in the process. The main feature which decides this is the removal of 5’deoxyribose-5-phosphate (5’dRP) upon insertion of the first nucleotide which occurs in AP sites. Polβ has lyase activity and can catalyse the release of 5’dRP from the AP sites by β-elimination. Oxidised/reduced AP sites are resistant to β-elimination because they do not have AP sites (5’ –dRP). Thus the decision between short-patch and long-patch BER is made ultimately by the glycosylase activity on the AP site. Short-patch BER has a regular AP site and the process is followed by DNA polymerase β and ligase I or DNA ligase III/XRCC1. Long-patch BER has an oxidised or reduced AP site in which the following processes may be involved in repair of this site. The process is completed by polymerase α, ε or β and PCNA/RFC, followed by FEN1, PCNA and DNA ligase 1 (Hansen and Kelly, 2000). The final step of the BER pathway is ligation with the DNA ligases mentioned. It was estimated that only 25% of lesions are repaired via the long-patch repair pathway (Dianov et al, 1998).

No human syndromes have been attributed to defects in BER to date and it is postulated that perhaps defects in BER may be lethal to the cell. However, single-nucleotide polymorphisms in a gene called MYH which encodes a glycosylase that
recognises 8-oxoG (which is repair through the short-patch pathway) have been linked
in increased susceptibility to colorectal cancer (Cline and Hanawalt, 2003).

2.2 Nucleotide excision repair (NER)
Nucleotide excision repair is the main strategy for repairing damage mainly derived
from exogenous sources. UV and chemical exposure can damage the base structure
of DNA from large chemical additions or crosslinks forming bulky DNA lesions.
NER is employed to repair such damage through excision of the short, single-stranded
segment which contains the damage (Cline and Hanawalt, 2003).
There are two subpathways of NER termed global genomic repair (GGR) and
transcription-coupled repair (TCR). GGR is the repair process of lesions throughout
the genome, whereas TCR is selective for the transcribed DNA strand in expressed
genes (Hanawalt et al, 2003).
TCR was first described by Philip Hanawalt in the mid-1980's where they noticed that
the rate of NER in mammalian genes that are actively transcribed is faster than those
that are in transcriptionally silent regions (Friedberg, 2001). The detailed pathway of
TCR is not yet understood. However, the prevailing model is subserved by the
arrested RNA polymerase II transcription machinery. This arrested complex is
thought to include additional proteins such as CSA and CSB (associated with a
disorder known as Cockayne syndrome). Some of the proteins that participate in TCR
have been elucidated (Svejstrup, 2002).
The repair pathway of GGR can be divided into four different steps:
recognition/preincision, incision, gap-filling and structural repair. Recognition of the
damage occurs from a protein complex comprised of XPC/HR23B and the UV
damage binding protein UV-DDB. For some damage the complex can efficiently
recognise the damage without UV-DDB (Hanawalt et al, 2003). A third protein known as centrin2/caltractin1, which is present in the centrosomes of several organisms has recently been shown to aid the stabilization of XPC to HR23B (Araki et al, 2001). XPA (which is a metalloprotein and is known to bind to many types of DNA damage in vitro) and RPA (which is a single-stranded DNA binding protein) are then recruited to verify the DNA damage. After recognition of the damage, the NER factors are assembled to facilitate the process. Among these factors is a subcomplex called core transcription factor IIH (TFIIH) which is composed of 6 subunits, constituting an RNA polymerase II to initiate the transcription process (Friedberg, 2001). The DNA helicases XPB and XPD are also components of the TFIIH complex and they facilitate transcription processes and the unwinding of the DNA helix in response to DNA damage generating junctions between double-stranded and single-stranded DNA. Two nucleases, XPG and the heterodimeric ERCC1-XPF are then recruited to cut the specific sites. XPG for cutting the strand 3’ to such sites and ERCC1-XPF for cutting the strand 5’ to damaged sites. A 24- to 32-residue oligonucleotide is released and the gap is filled in by polymerase δ or ε holoenzyme and is then subsequently sealed by a DNA ligase, probably LIGI (Lindahl and Wood, 1999).

It is known that defective NER predisposes to cancer. NER defects with the inherited human autosomal recessive disorder Xeroderma pigmentosum (XP) are caused by mutations in XP genes. Patients with XP are sensitive to UV damage and show increased incidence rates of skin cancer. James Cleaver and Richard B. Setlow in the 1960’s were the first to demonstrate that cells from patients with XP have defective NER (Friedberg, 2001). Furthermore XP genes are controlled in a P53 dependent
manner which has implications for the carcinogenesis process (reviewed by Hanawalt et al, 2003).

Another human syndrome known as Cockaynes syndrome (CS) is also associated with defective NER. CS patients are characterised by severe sensitivity to UV radiation and congenital neurological and skeletal abnormalities. Defects in CSA and CSB genes are responsible for this syndrome. These proteins were proposed to be involved in transcription-coupled NER as mentioned previously (Lindahl et al, 1997). Cancer predisposition phenotype is not associated with this disease.

2.3 Mismatch repair (MMR)

Mismatch repair is a strategy responsible for repairing base mismatch DNA damage derived from both endogenous and exogenous factors. Base mismatches include mispaired nucleotides and insertion/deletion loops and they occur from processes such as deamination, oxidation, methylation and replication errors which occur spontaneously or are induced (Christmann et al, 2003).

The pathway of MMR is divided into three main groups: recognition, incision and repair synthesis. Recognition of the damage is carried out through the MutSα complex which binds to the damaged site. This is comprised of proteins known as MSH2 and MSH6. MSH2 can form a complex with MSH3 and this has been termed the mutSβ complex. The mutSα complex binds to base-base mismatches and small loops derived from insertion/deletion mismatches whereas the mutSβ complex binds only to larger loops from insertion/deletion mismatches (Cline and Hanawalt, 2003). MutSα is subsequently bound to hMutLa complex which is comprised of hMLH1 and hPMS2 proteins. The DNA strand with the mismatched base is excised by
exonuclease I and repaired by polymerase δ. Subsequent ligation occurs to seal the helix.

Defects in MMR associated genes have been associated with cancer predisposition. It was demonstrated by Fishel and Wilson (1997) that mutations in either MSH2 or MLH1 genes are estimated in over 90% of HNPCC cases. Defects in mismatch repair genes have also been associated with other cancers such as endometrial cancer (Berends et al, 2003), ovarian cancer (Geisler et al, 2003) breast cancer (Balogh et al, 2003) and other cancers such as gastric, urologic tract, small bowel and hepatoliliary tract carcinomas and brain tumours (reviewed by Watson and Lynch, 2001).

2.4 Direct reversal of base damage

This is a method of repair which removes the alteration to the base without removing the damaged base and it is thought to be the most efficient mechanism of repair for this reason (Hansen and Kelley, 2000). The main mode of action of direct reversal is alkylation damage which can cause misparing damage of GC to AT during the replication process. Some forms of alkylation damage can be repaired by the NER pathway mentioned previously, however the NER pathway is inhibited in mammalian cells by binding to methyltransferases (Samson et al, 1997) and other repair processes such as direct reversal is required.

Proteins that are involved in the removal of alkyl groups from the O\(^6\) position of guanine and the O\(^4\) position of thymine are known as alkyltransferases. One associated human protein was described originally by Tano et al (1990). It was called O\(^6\)-methylguanine-DNA methyltransferase (MGMT) (Hansen and Kelley, 2000). It operates in a single-step repair process by transferring the methyl or chloroethyl group from the alkylated guanosine onto an internal cysteine residue in a conserved region
of the protein and it accepts the alkyl groups from the modified nucleotide. This activity leads to irreversible inactivation of the MGMT protein and targets it for degradation processes (Christmann et al, 2003).

Three additional proteins associated with direct reversal of base damage repair are known. These are ABH1, ABH2 and ABH3 and they are homologous to the AlkB proteins in bacteria. They are dioxygenases, as they function by catalysing the oxidation and release of the methyl group from 1-methyladenine and 3-methylcytosine and the ethyl group of 1-ethyladenine and subsequent restoration of the undamaged base.

MGMT levels in cancer cells have been shown to correlate to their sensitivity to chemotherapeutic agents. Increased MGMT levels have been observed in melanoma, ovarian, breast, lung, pancreatic, colon cancers and brain tumours (Hansen and Kelley, 2000). MGMT overexpression protects from tumour initiation and progression upon exposure to alkylating agents (Becker, 2003).
1.7 CELL DEATH AS A RESULT OF DNA DAMAGE

In the previous sections, we have discussed the underlying mechanisms of the cell cycle for normal control (the basis for the decision to divide or to differentiate) and its activities in response to genetic alterations including DNA repair mechanisms. However, cell death is another important mechanism which may be activated intrinsically by the cell or induced by DNA damage. A clear distinction between the processes underlying the death of cells during embryogenesis and tissue kinetics at a microscopic level was first established by Kerr et al (1972). A cell death process known as necrosis was characterised by general cell death induced by physical or chemical mutagen exposure to the cell. It has also been described as a pathological process which occurs as a consequence of impaired membrane transport with uncontrolled movement of ions and fluids (Cardoso and Leonhardt, 1999). Apoptosis or programmed cell death is another cell death mechanism which occurs during embryonic development, normal tissue turnover (regulating the number of each cell type), and endocrine tissue atrophy and thus, is the most important death mechanism. Many studies have been conducted in recent years that have elucidated the molecular basis of apoptosis. Genetic and biochemical experiments have indicated that apoptosis is controlled by the Bcl-2 family of proteins, and these proteins may act as agonists (Bax or Bak) or antagonists (Bcl-2 or Bcl-XL) of the apoptosis, and the balance between these two factors determines whether the cell lives or dies (Cardoso and Leonhardt, 1999). These factors can dimerise with each other and the predominance of Bax homodimers activates a cascade of ICE (Interleukin-1β-converting enzyme) proteases cleaving intracellular substrates which cause the morphological and biochemical changes of apoptosis. Apoptosis is an important
process in cancer formation, as the accumulation of neoplastic cells can occur through enhanced proliferation and/or diminished cell turnover (Evan and Vousden, 2001). The Bcl-2 oncogene was identified as an important factor in this process as it blocks apoptosis and promotes neoplastic formation. Alterations of expression of Bcl-2 oncogene have been shown to be associated with tumour development (See section 1.2.1 on oncogenes). Bcl-2 oncogenes have also been shown to interact with other oncogenes such as c-myc in cancer development by blocking c-myc induced apoptosis (Evan and Vousden, 2001).

P53 tumour suppressor gene plays a vital role in apoptosis and cancer development. It was the first tumour suppressor gene to be linked to apoptosis (Vogelstein et al, 2000). See section 1.2.2. Mutations in P53 have been implicated in the majority of human cancers.

Apoptosis and Necrosis can be induced by physical and chemical mutagen exposure, and this has been demonstrated in abundant cell studies. Apoptosis has been shown to be cell type dependent as a radiation induced cell death mechanism with hemopoietic and lymphoid cells particularly prone to apoptosis post ionising radiation exposure (Hall, 2000). Mitotic death has recently been described as a cell death mechanism associated with post-irradiation mitosis (Hall, 2000). The cells die in attempting to divide because of damaged chromosomes. Mitotic death is the most common form of cell death associated with ionising radiation exposure and it is described as a delayed cell death mechanisms induced by IR. Cells exposed to IR are usually halted at G2 as the first line of defense, however Andreassen et al (2003) suggested that the inability to maintain arrested cells in G2 means that damaged cells can adapt and progress in the cell cycle leading to mitotic errors. Erenpreisa et al (2003) proposed the initiation of an endocycle from G2 arrest in the formation of these mitotic errors and
subsequently delayed reproductive death or mitotic death occurs. It was postulated that cells which are exposed to low LET radiation may undergo death through apoptosis or mitotic death in contrast to cells which are exposed to high LET radiation which may undergo death from necrosis (reviewed by Harms-Ringdahl et al, 1996). Thus, the radiobiological studies carried out throughout this thesis used only low dose low LET radiation and therefore the cell death mechanisms of apoptosis and mitotic death are the most relevant. A recent study by Howe et al (manuscript submitted, 2005) demonstrated an inverse correlation of mitotic errors with G2 radiosensitivity, which indicate that apoptotic and mitotic cell death mechanisms may be occurring in lymphocytes of prostate cancer patients due to low LET radiation exposure. This data strengthens the existing hypotheses in the literature. See chapter 3 for a more detailed description of this data.

1.8 SUMMARY OF THESIS OBJECTIVES

1. To use and assess various assays for detecting the sensitivity of various human genetic disorders to mutagens which cause DNA single strand or double strand breaks.

2. To investigate the genetic mechanisms that may be responsible for the sensitivity phenotype among patients with various human genetic disorders.

3. To elucidate the underlying cellular and molecular pathways that may occur in relation to mutagen sensitivity in these patients.
CHAPTER 2

THE G2 CHROMOSOMAL RADIosenSITIVITY ASSAY FOR THE IDENTIFICATION OF A RADIosENSITIVE SUBPOPULATION AMONG IRISH BREAST CANCER PATIENTS

2.1 Introduction
2.2 Materials and Methods
2.3 Results
2.4 Discussion
2.1 INTRODUCTION

The acquisition of the malignant phenotype in breast carcinomas is a progressive and cumulative effect of altered genotypes. Altered genotypes (mutations) can be inherited or occur spontaneously and can then contribute to cancer predisposition. Various high-penetrance and low-penetrance cancer predisposition genes have been identified in both hereditary and sporadic breast cancers. Linkage analysis studies have identified two important highly-penetrant breast cancer susceptibility genes, BRCA 1 and BRCA 2 mapped to chromosome 17q21 (Hall et al, 1990) and 13q12-13 (Wooster et al, 1995) respectively. These genes were thought to account for 65-80% of hereditary breast cancer and <5% of all breast cancer (Easton et al, 1995, Gayther et al, 1998). Mutations of these genes are rarely found in sporadic breast cancers which make up approximately 90% of all breast cancer cases (Neville et al, 2001). Other highly penetrant genes such as those responsible for rare genetic syndromes have been shown to be associated with breast cancer and risk. These include the Tp53 gene (li-fraumeni syndrome) which has been found in breast cancer (Harris and Holstein, 1993) of which inherited mutations are thought to account for less than 1% and somatic mutations accounting for 19-57% (de Jong et al, 2002). Gene such PTEN/MMAC1/TEP1 gene (Cowdens syndrome) and STK11/LKB1 gene (Peutz-Jeghers syndrome) have also been implicated in breast cancer risk (Arver et al, 2000).

Low-penetrance genes have also been identified and associated with breast cancer. ATM from the autosomal recessive human disorder Ataxia-Telangectasia is one of these genes and was first reported by Swift et al (1987). The relative risk of breast cancer in AT heterozygotes varies from approximately 4% (Easton, 1994) to 12.7% (Stankovic et al, 1998) and this risk factor was reviewed by Angele and Hall (2000).
It was postulated that among low-penetrance predisposition genes are those involved in cell cycle checkpoints and DNA repair processes in response to DNA damage, and when defective lead to enhanced radiosensitivity. The link between breast cancer genes and DNA damage has recently been shown. It was postulated that BRCA1 and BRCA2 are involved in DNA Damage pathways possibly through regions of the protein products of these genes such as the carboxyl terminus (BRCT domain) of BRCA1 which subsequently binds to other proteins such as the repair protein XRCC1 and initiates a signalling cascade in response to DNA damage (Caldecott, 2003). It was recently reported that BRCT binds to a DNA repair helicase BACH1 which is phosphorylated during the G2/M phase of the cell cycle and is therefore required for the G2 checkpoint in the DNA damage response (Yu et al., 2003). A mutation (1100delC) in the cell cycle checkpoint gene (CHEK2) has recently been described in breast cancer patients that do not have mutations in BRCA1 or BRCA2 (Meijers-Heijboer et al., 2002). It is thought that this mutation doubles the risk of breast cancer among women and increases the risk factor in male breast cancers to a factor of ten. Low-penetrance DNA repair genes XRCC1, XRCC3 and XRCC5 were shown to exhibit enhanced radiosensitivity when mutated (Price et al., 1997). However it was hypothesised by Terzoudi et al. (2000) that upset of the mechanisms regulating cell cycle control (cdk/cyclinB activity) underlies increased G2 radiosensitivity rather than deficient DNA repair enzymes.

The genetic mechanisms of cell cycle and DNA repair processes in response to DNA damage and their association with breast cancer has been demonstrated. It is postulated that radiation-induced G2 chromosomal radiosensitivity could be a marker
of some of these breast cancer predisposition genes of low penetrance related to cell
cycle and DNA repair.

Several independent research groups have carried out the G2 chromosomal
radiosensitivity assay as a predictive test for low-penetrance predisposition genes on
patients with a variety of disorders. Earlier G2 studies (predominantly from the
National Cancer Institute in Bethesda) were carried out on fibroblast cell lines from
patients with Ataxia-Telangiectasia (Shiloh et al, 1989), Bloom syndrome, Fanconi
anaemia, Gardner’s syndrome and Xeroderma Pigmentosum where enhanced G2
radiosensitivity was recorded (Sanford and Parshad, 1990, 1985, 1984, 1983). The G2
assay was also applied to lymphoblastoid cell lines (Lavin et al, 1994).

For a test to be useful for clinical screening it must essentially be blood or urine
based, with easy application and must generate results as quickly as possible. The
application of the G2 assay to lymphocytes from whole blood samples extracted from
patients with the various syndromes described above is such a method (Sanford et al,
1990). Other conditions such as breast cancer (Baria et al, 2002, Scott et al, 1999,
(Baria et al, 2001), head and neck cancer (Papworth et al, 2001) have also been
studied in this way.

Elevated sensitivity has been shown in approximately 40% of UK breast cancer
patients in comparison to 10% of the normal control population using the G2 assay
(Scott et al, 1994, 1999). These levels of radiosensitivity have also been shown in the
G0 phases of the cell cycle using the micronucleus assay which is also a cytogenetic
based assay. However, no correlations were shown between G2 and G0
radiosensitivity (Burrill et al, 2000) in breast cancer patients.
Previous G2 radiosensitivity assay studies also demonstrated that 50% of first-degree relatives of the radiosensitive patients (i.e. exhibiting a high G2 score) also showed radiosensitivity levels suggesting that a cancer predisposition gene or genes related to the heritability of cellular radiosensitivity existed within certain families (Roberts et al., 1999).

Various enhanced G2 radiosensitivity levels in breast cancer patients have been described by international laboratories at the National Cancer Institute (NCI), Bethesda, USA. The Paterson Institute of Cancer Research, UK and the Queensland Institute of Medical Research (QIMR), Australia. Increased G2 radiosensitivity levels varied from 18% (QIMR) to 40% in (PICR).

Chromosomal radiosensitivity is therefore an important biomarker for breast cancer susceptibility genes of low penetrance, and may vary between geographic locations. This study aim is to identify the level of G2 radiosensitivity among breast cancer patients, within the Irish population, and to compare this level to existing international data. A long term objective is to assess the potential use of the G2 assay as a predictive test for breast cancer predisposition particularly those with a possible inherited predisposition not related to mutations in BRCA 1 or 2.
2.2 MATERIALS & METHODS

2.2.1 Patients and control samples

Blood samples from 27 breast cancer patients eligible for the study were taken and analysed. Patients were not on active therapy at the time of sampling. The age group of the patients ranged from 34 - 77 years (mean age was 60 years). Eligibility criteria for the study entailed that patients who had received radiotherapy were used on the provision that they were sampled at least 6 months after their treatment to allow lymphocyte recovery. Four patients who had received prior chemotherapy were included in the study. See Table 2.1 for a brief synopsis of the patients’ histories.

The patients were defined as sporadic cases.

Ethical approval for this study was given by the Federated Dublin Voluntary Hospital and St. James’s Hospital Joint Research Ethics Committee and patients gave written, informed consent prior to participation.

Inter-control variation and intra-control variation analyses were carried out on blood samples obtained from a control population to standardise the G2 assay for our Dublin laboratory and to ensure assay reproducibility. Variance was expressed as coefficient of variation (CV).

10 blood samples were taken from 10 random control subjects for inter-control variation and 10 repeat blood samples were taken from one control source at different intervals for intra-control variation. These samples were taken and transported with the breast cancer patient samples.
### Table 2.1 Breast Cancer Patient History

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Age</th>
<th>Prior therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2 – S1</td>
<td>69</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S2</td>
<td>39</td>
<td>CMF (1999)</td>
</tr>
<tr>
<td>G2 – S3</td>
<td>58</td>
<td>CMF (1991)</td>
</tr>
<tr>
<td>G2 – S4</td>
<td>34</td>
<td>CMF (1997)</td>
</tr>
<tr>
<td>G2 – S5</td>
<td>40</td>
<td>CMF (1985)</td>
</tr>
<tr>
<td>G2 – S6</td>
<td>55</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S7</td>
<td>68</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S8</td>
<td>58</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S9</td>
<td>55</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S10</td>
<td>66</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S11</td>
<td>67</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S12</td>
<td>73</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S13</td>
<td>57</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S14</td>
<td>77</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S15</td>
<td>65</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S16</td>
<td>77</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S17</td>
<td>54</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S18</td>
<td>74</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S19</td>
<td>49</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S20</td>
<td>44</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S21</td>
<td>69</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S22</td>
<td>75</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S23</td>
<td>52</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S24</td>
<td>67</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S25</td>
<td>55</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S26</td>
<td>63</td>
<td>NA</td>
</tr>
<tr>
<td>G2 – S27</td>
<td>58</td>
<td>NA</td>
</tr>
</tbody>
</table>

MEDIAN AGE: 58 yrs (range 34-77). NA- Not Applicable.

#### 2.2.2 The G2 Assay

The G2 method used for this study was the PICR G2 protocol (Scott et al, 1996).

The method is summarised here:
Lymphocyte cultures were set up using a total volume of 8mls of whole blood divided between 4 flasks (Corning), 2 x control flasks and 2 x irradiated flasks. 2ml of blood was used per flask, 200μl of mitogen, phytohaemagglutinin (PHA) (Miurex HA-15) and 18ml of RPMI 1640 medium (Sigma) supplemented with 12.5% foetal calf serum (Gibco) and 2mM L-Glutamine (Gibco). The cultures were mixed well and incubated in an upright position at 37°C with 5% CO₂. 48 hours later the culture medium was changed by pipetting 15mls of medium carefully out of each flask (taking care not to disrupt the unattached layer of cells) and pipetting in 15mls of fresh, pre-warmed and pre-gassed RPMI medium (as above) and 150μl PHA. 24 hours later, the cells were irradiated to 0.5Gy using the cobalt 60 gamma ray teletherapy unit at St. Luke’s Hospital, Dublin. The control flasks were sham-irradiated. The flasks were transported in a 37°C portable incubator 30 minutes prior to, and post irradiation. The cultures were incubated with 200μl of colcemid 1mg/ml (Gibco) for 60 minutes to halt the cells in metaphase 30 minutes post irradiation.

The contents of each flask were split between two centrifuge tubes per sample flask and they were plunged into ice. They were then centrifuged (Sorvall) at 1200rpm for 5 minutes. The supernatant was pipetted off with a disposable Pasteur pipette (Aldrich) and 5ml of 0.075M KCl (BDH) was added to the pellet of cells. The cells were incubated on ice for 20 minutes. The cells were centrifuged at 1200rpm for 5 minutes. The supernatant was pipetted off and 3:1 methanol (BDH):acetic acid (BDH) fixative was added to the cells. This step was repeated and the cells were left overnight or longer at 4°C. For slide-making, the cells were allowed to stand at room temperature before centrifugation. Fresh fixative was made and two further fixation steps were carried out on the cells as above (to completely clean the cells). The cells
were added dropwise on to slides pre-cleaned in methanol. The slides were left overnight to dry completely. The slides were stained with 2% Giemsa solution (Gibco) prepared in pH 6.8 buffer (BDH) for 10 minutes and mounted with DPX.

2.2.3 Metaphase Analysis – G2 Score

The slides were coded and random metaphases were scored on the slides. Vernier readings of each metaphase scored were recorded to ensure that there was no overlap in metaphase scoring. Fifty well-spread and clear metaphases per sample were scored. The number and type of aberrations were recorded per 50 metaphases expressed as a percentage. The bulk of aberrations constituted chromatid gaps and breaks. Acentrics and chromatid minutes were recorded on rare occasions. Tetraploidy and endoreduplication were recorded and classified separately. Cells that had more than double the number of normal aberrations in one metaphase for that particular slide were considered rogue cells and were not included in the G2 score.

2.2.4 Statistical Analysis

Inter and intra-individual variations of the control samples were quantified by a one-way analysis of variance. The variances were expressed as coefficient of variations. The G2 score was expressed in terms of a sensitive/non-sensitive dichotomy, and the G2 radiosensitive cut-off point was calculated as the 90th percentile of results (Scott et al, 1998). The non-parametric Mann-Whitney U-test was used to compare the control group with the breast cancer patient group. Correlations of parameters within the breast cancer patient group were carried out using Spearman’s rank correlation coefficient. A significance level (p) of 0.05 was used throughout.
2.3 RESULTS

2.3.1 G2 assay standardisation and reproducibility

The radiosensitive G2 scores were calculated by subtracting the spontaneous aberrations (non-irradiated) (figure 2.1) from the radiation-induced aberrations (irradiated) (figure 2.2) per subject. The mean values (μ), the standard deviation (SD) and the coefficients of variation (CV=SD/μ x 100) were also calculated per donor group of sample and are illustrated in table 2.2. As a measure of assay reproducibility, the intra-individual and inter-individual variation was calculated on a random control population. The G2 assay was carried out on 4 donor controls (coded D1-D4) to measure intra-individual variation. 5 or 10 samples were taken from each of these donors at various intervals. The G2 assay was carried out on single blood samples from 10 random donor controls to measure inter-individual variation. The variation (CV) for the intra-individual donors D1-D3 was between 4.58%-5.09%, however the variation for D4 was 22.94%. The chi-squared test was performed on all of these donors (D1-D4) and showed that there was a significant difference in the values between the groups (p ≥ 0.001). The chi-squared test was carried out on donors (D1-D3) and the difference between them was considered not significant (p ≥ 0.990). This indicates that the variation values from donors D1-D3 were more representative of intra-individual variation than D4. The CV for inter-individual variation was calculated at 17.03%. The variance-ratio F test was used to compare the intra-individual variances (from D1-D3) with the inter-individual variance and they were shown to be not significantly different.
Table 2.2: Statistical evaluation of the G2 radiosensitivity scores from the intra-variation controls and inter-variation controls compared to breast cancer patients.

<table>
<thead>
<tr>
<th>Donor group</th>
<th>No of samples</th>
<th>Mean ((\mu))</th>
<th>S.D.</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-variation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>5</td>
<td>103.6</td>
<td>5.18</td>
<td>5.00</td>
</tr>
<tr>
<td>D2</td>
<td>5</td>
<td>75.60</td>
<td>3.85</td>
<td>5.09</td>
</tr>
<tr>
<td>D3</td>
<td>10</td>
<td>96.60</td>
<td>4.43</td>
<td>4.58</td>
</tr>
<tr>
<td>D4</td>
<td>10</td>
<td>172.22</td>
<td>39.50</td>
<td>22.94</td>
</tr>
<tr>
<td><strong>Inter-variation</strong></td>
<td>10</td>
<td>86.16</td>
<td>14.68</td>
<td>17.03</td>
</tr>
<tr>
<td><strong>Breast cancer</strong></td>
<td>27</td>
<td>142.96</td>
<td>36.96</td>
<td>25.85</td>
</tr>
</tbody>
</table>

2.3.2 Comparison of control group versus breast cancer patient group

It is important to analyse the G2 scores obtained from all patient samples in the G2 radiosensitivity assay in terms of a sensitive/non-sensitive dichotomoy. A reasonable method of statistically defining the radiosensitivity cut-off value is to calculate a 90th percentile of below which 90% of the controls lie (Scott et al, 1999). A cut-off value of 110 aberrations/100 metaphases was calculated from all of the control subjects (n=13) using the inter-variation donors and the 3 intra-individual variation donors D1-D3 (using a mean G2 score for the donor). Using this cut-off value 7.7% (1/13) of
the control population was calculated as G2 radiosensitive in contrast to 70.4% (19/27) of the breast cancer population. Figure 2.1 shows the distribution of the G2 scores in the controls compared to the breast cancer patients. The G2 radiosensitive values of the breast cancer patients were sub-classified into a high and low G2 radiosensitivity range (HRR and LRR) according to the median value (176 aberrations/100 metaphases) between the maximum and minimum G2 radiosensitive scores (110 and 242 aberrations respectively). 26% (5/19) of the G2 scores were in the HRR compared to 74% (14/19) which were in the LRR.

2.3.3 Correlations among breast cancer patients

4/27 of the breast cancer patients had chemotherapy prior to sampling. The G2 scores of the four chemotherapy patients were compared to the 23 other patients. Although the overall mean aberration frequency of the patients with chemotherapy exposure (174 ± 50.8 SD, median 167) was higher than the mean aberration frequency of the patients without chemotherapy (138.1 ± 32, median 130), the difference between them was considered not significant (The two-tailed p-value was exact at 0.1680). The Spearman rank correlation coefficient also showed that the association between the G2 scores of the patients who received chemotherapy and the time elapsed since they received it was not significant (p value = 0.7500). As a result, the data from all 27 patients were analysed together.
**Figure 2.1:** Unirradiated lymphocyte metaphase showing 46 fully-intact undamaged chromosomes.

**Figure 2.2:** Irradiated lymphocyte metaphase showing chromatid damage. (CTG – chromatid gaps, CTB – chromatid breaks)
Figure 2.3: Distribution of G2 radiosensitivity scores in normal healthy controls compared to breast cancer patients.

Ranges of G2 scores in normal healthy control population

<table>
<thead>
<tr>
<th>Range (aberrations per 100 metaphases)</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-80</td>
<td>1</td>
</tr>
<tr>
<td>80-100</td>
<td>3</td>
</tr>
<tr>
<td>100-120</td>
<td>2</td>
</tr>
<tr>
<td>120-140</td>
<td>4</td>
</tr>
<tr>
<td>140-160</td>
<td>3</td>
</tr>
<tr>
<td>160-180</td>
<td>2</td>
</tr>
<tr>
<td>180-200</td>
<td>1</td>
</tr>
<tr>
<td>200-220</td>
<td>1</td>
</tr>
<tr>
<td>220-240</td>
<td>0</td>
</tr>
<tr>
<td>240-260</td>
<td>1</td>
</tr>
</tbody>
</table>

G2 radiosensitivity cut-off value

Ranges of G2 scores in breast cancer patients

<table>
<thead>
<tr>
<th>Range (aberrations per 100 metaphases)</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-80</td>
<td>1</td>
</tr>
<tr>
<td>80-100</td>
<td>3</td>
</tr>
<tr>
<td>100-120</td>
<td>2</td>
</tr>
<tr>
<td>120-140</td>
<td>4</td>
</tr>
<tr>
<td>140-160</td>
<td>3</td>
</tr>
<tr>
<td>160-180</td>
<td>2</td>
</tr>
<tr>
<td>180-200</td>
<td>1</td>
</tr>
<tr>
<td>200-220</td>
<td>1</td>
</tr>
<tr>
<td>220-240</td>
<td>0</td>
</tr>
<tr>
<td>240-260</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.3: Previous G2 radiosensitivity studies carried out in breast cancer patients

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>% Radiosensitivity</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scott et al</td>
<td>1994</td>
<td>*42%</td>
<td>Lancet, 344, 1444.</td>
</tr>
<tr>
<td>Baria et al</td>
<td>2001</td>
<td>*40%</td>
<td>Br.J.Cancer, 84, 892-896.</td>
</tr>
<tr>
<td>Riches et al</td>
<td>2001</td>
<td>*46.2%</td>
<td>Br.J.Cancer, 85, 1157-1161.</td>
</tr>
</tbody>
</table>

* The % radiosensitivity in breast cancer patients calculated from the G2 scores in the studies reported to date.
2.4 DISCUSSION

The results from the present study confirm that a significant proportion of breast cancer patients exhibit elevated G2 chromosomal radiosensitivity in contrast to random controls. The Irish patients used for this study were sporadic breast cancer patients and specifically confirm the enhanced sensitivity recorded in sporadic breast cancer patients from several independent studies (Scott et al, 1994; 1999; Terzoudi et al, 2000; Riches et al, 2001). It was initially estimated by Scott et al (1994; 1999) that the proportion of G2 radiosensitive breast cancer patients was approximately 40% in contrast to approximately 10% recorded in controls. Several studies have shown similar estimates (46.2% Riches et al, 2001; 43% Baeyens et al, 2002). In this study we calculated 70.4% G2 radiosensitivity in the Irish breast cancer patients in comparison to 7.7% G2 radiosensitivity in controls. This G2 value is higher than those seen in other studies which varied from 39%- 46.2% displayed in table 1. This may be due to the fact that only 27 patient samples were used for the study and so it was therefore concluded that a larger number of samples would be needed to consolidate our higher estimate.

Due to the high % G2 radiosensitivity value obtained for the breast cancer patients, their G2 scores were sub-classified into a high and low radiosensitivity range (HRR and LRR respectively) of values based on the median between the radiosensitive G2 scores. It was estimated that the bulk of the G2 radiosensitivity scores were in the LRR (74%) compared to the HRR (26%) which suggests that if the radiosensitivity cut-off value (90th percentile) of the controls was higher then the overall G2 radiosensitivity value would be reduced. This has led us to conclude that the small number of control samples (n=13) used in our study may be an important factor in this
high G2 radiosensitive value and that a larger number of control samples in an extended study would consolidate our data.

Although the patient and control sample numbers in this study were small in comparison to other studies, and may have affected our overall % G2 radiosensitivity estimate in breast cancer patients, the G2 assay was standardized through the assessment of procedures and tested for assay reproducibility. The protocol of Scott et al (1999) was used and with their assay they showed good intrinsic assay reproducibility with the intra-individual variation between 7-10% (Scott et al, 1999; Papworth et al, 1999) and inter-individual variation approximately 20% with this protocol. The only adjustment made to this protocol for our study was that the blood samples were stored up to 24 hours at ambient temperature before use as this was an acceptable practice by the various groups doing the G2 assay (Bryant et al, 2002) and previous reports showed that this has no effect on aberration yields (Scott et al, 1999; Smart et al, 2003). The scoring criterion was also strictly adhered to despite conflicting views of scoring chromatid gaps (big and small) raised at a workshop at St. Andrew’s University. (Bryant et al, 2002). There was considerable debate regarding what aberrations should be recorded to contribute to the G2 score. Thus, it was necessary to form a definition of the aberrations found in this assay. Chromatid breaks were defined as chromatid discontinuties with displacement or misalignment of the chromatid fragment. Chromatid gaps were defined as lesions showing no dislocation in spite of chromatid discontinuity and were scored only if longer than the chromatid width (Sanford et al, 1989; Scott et al, 1998). It was concluded at the end of the workshop that all discontinuties regardless of size, should be scored as gaps. However, this research work had begun before the workshop and the PICR protocol
was adhered to rigidly throughout the study to minimise variation between the results. Thus, small gaps were scored for the remainder of the study but they were recorded separately and were not included in the final G2 scores.

The inter-individual variation was calculated at 17.03% which was below the expected value described above. In three out of four of the donors that were used for intra-individual variation, the calculated coefficients of variation were between 4.58% - 5.09% thus indicating good intrinsic assay variability and are also below the expected range described above. Furthermore, the chi-squared test showed that there was no significant difference between these donor groups (p ≥ 0.990). One of the four donors showed a variation of 22.94% and when this was compared to the other three donor groups with the chi-squared test, a significant difference was observed (p ≥ 0.001). We concluded that this high intra-individual variation in this donor was not due to experimental procedures but may be an intrinsic factor within the individual. It is thought that factors such as diet and hormone levels may have some influence on cytogenetic endpoints used for chromosomal radiosensitivity assessment (Roberts et al, 1997). Problems associated with intra- and inter-individual variation have previously been documented (Vral et al, 2002; 2004; Smart et al, 2003) and it was concluded that although the G2 assay is a useful tool for examining population radiosensitivity, assessment of individual radiosensitivity should not be based on a single blood sample and that multiple blood samples may be necessary to draw reliable conclusions. More studies to validate the reliability of the G2 assay are required but it is well known that the assay is a useful biomarker of radiosensitivity in populations and of cancer predisposition genes of low penetrance. The exact mechanisms which are involved in the formation of G2 chromatid breaks have not
been elucidated but different hypotheses have been proposed in various studies (Parshad et al, 1996; Bryant, 1998; Terzoudi et al, 2000). It has been generally accepted that G2 radiosensitivity is involved in the processing of DNA damage and when these genes are defective cause this elevated G2 sensitivity (Parshad et al, 1993; Scott et al, 1999). If these low-penetrance genes associated with G2 radiosensitivity could be identified in the future, they could serve as markers for breast cancer predisposition and be used for population screening. The G2 assay could also be used for population screening but it would have to be on a smaller number of patients as it is very time-consuming and technically demanding for large studies.
CHAPTER 3

THE ASSESSMENT OF IN VITRO TECHNIQUES AS POTENTIAL BIOMARKERS FOR PREDICTING RADIOSensitivity IN PATIENTS WITH BENIGN PROSTATIC HYPERPLASIA AND PROSTATE CANCER

3.1 Introduction
3.2 Materials and Methods
3.4 Results
3.5 Discussion
3.1 INTRODUCTION

Prostate cancer and benign prostatic hyperplasia (BPH) are common diseases in men, and are associated with risk factors such as age and hormonal imbalance. Both diseases are related to the uncontrolled growth or proliferation of cells but prostate cancer is a malignant condition in contrast to BPH which is benign. Both have specific aetiological factors contributing to the disease.

In recent years, specific gene products and genetic pathways have been identified which elucidated some of the underlying molecular mechanisms that contribute to normal prostatic development and diseases. Genes associated with normal prostatic development have been identified through several in vivo and in vitro cell culture systems. Manipulation of primary prostate cultures has demonstrated that two fibroblast growth factors (FGF); FGF7 and FGF10 are expressed in the developing prostate and act through a FGF receptor (FGFR2) to promote epithelial proliferation and therefore promote prostatic dual morphogenesis (Sugimura et al., 1996; Thompson and Cunha, 1999). Additional factors such as the secreted activin A (bound to follistatin) and hyaluronan (bound to CD44) are implicated in prostatic morphogenesis and fucosyltransferase1 transmembrane enzyme was implicated in prostatic development (reviewed by Marker et al, 2003). Murine models have also elucidated genes associated with androgen dependence in normal prostate development. Two genes; Srd5a1 and Srd5a2 encoding the enzyme 5-α reductase type 1 and type 2 were identified. These enzymes catalyse the conversion of testosterone to a more potent androgen dihydrotestosterone (DHT) (Russell and Wilson, 1994). However, testosterone has a higher affinity for type 2 making it more the more dominant type. The Akr1c4 gene encodes 3α-hydroxysteroid dehydrogenase (3α-HSD) and operates in a similar manner by catalysing the conversion of circulating
5α-adiol to DHT. These genes cause the upregulation of Shh (Podlasek et al, 1999a) and Nkx3.1 (Sciavolino et al, 1997) in the molecular mechanisms relaying prostatic development and growth. This role of DHT in normal prostatic development and growth has significant implications for the role of 5α-reductase inhibitors in treating benign prostatic hyperplasia where abnormal growth occurs (reviewed by Bartsch et al, 2002).

Benign prostatic hyperplasia is a common disease in men over 50 years of age, however little is known about its aetiology. The role of androgen dependence as a causative factor of BPH has been suggested through observations that patients with a variety of genetic diseases have impaired or absent prostatic growth. DHT is the principal prostatic androgen converted from testosterone as described above. It was demonstrated that DHT levels remain normal with increasing age in men despite the reduction in testosterone levels (Walsh et al, 1983). DHT is twice as potent as testosterone (Wright et al, 1999), it has a higher affinity for the androgen receptor than testosterone (Grino et al, 1990) and it accumulates in the prostate when testosterone levels are low providing an explanation for the DHT levels remaining normal with increasing age. DHT can accumulate and stimulate cellular proliferation; however the genes involved in this process and the exact mechanisms of androgen induced growth stimulation have not yet been elucidated. Studies including gene expression profiles using cDNA micro-array technology have been carried out for the identification of some of these associated genes in the pathogenesis of BPH (Luo et al, 2001; 2002).

Prostate cancer is a malignant disease associated with heterogeneous aetiologica factors including age, hormonal imbalance, lifestyle and diet. There is consistent evidence across racial and ethnic groups that genetic predisposition is also associated
with some prostate cancers (see chapter 1) (and reviewed by Schaid et al, 2004) and hereditary genetic factors in prostate cancer are believed to be as significant as breast and colon cancer (Lichenstein et al, 2000; Verkasalo et al, 1999). It was also predicted that approximately 42% of the risk of prostate cancer could be due to heritable factors (Lichenstein et al, 2000). Several of these genes have been identified through family-based linkage analysis (reviewed by Karan et al, 2003). The first putative locus for a prostate cancer gene was found at chromosome 1q24-25 and called HPC1 (Smith et al, 1996). PCAP mapped to chromosome 1q42.2-43 was the second putative loci reported (Berthon et al, 1998). A third locus; HPCX was mapped to chromosome Xq27-28 (Xu et al, 1998) followed by the identification of CAPB on chromosome 1q36 (Gibbs et al, 1999) and it was related to brain tumours in patients. The remaining putative loci for prostate cancer genes were HPC at 20q13 (Berry et al, 2000), HPC2/ELAC at 17p11 (Tavitigian et al, 2001) and finally HSD3B at 1p13 locus (Chang et al, 2002). Other loci such as; 16q23 (Suarez et al, 2000), 8p22-23 (Xu et al, 2001) and 20q13 (Bock et al, 2001) were also proposed as harbouring putative prostate cancer genes.

Mutations in other genes have also been associated with prostate cancer. It was proposed that BRCA1 and BRCA2 carriers have a lifetime risk factor of developing prostate cancer of 6% and 6-12% respectively (Ford et al, 1998). Mutations in CHEK2 have recently been identified in prostate cancer (Dong et al, 2003) but it was shown that they are low-penetrance prostate cancer predisposition alleles that contribute to familial clustering of prostate cancer in the population (Seppala et al, 2003). Prostate cancer has also been linked to heterozygous carriers of the A-T gene. A study carried out on prostate cancer patients with severe late responses to radiation therapy demonstrated that 18% had a significant mutation in the ATM gene (Hall et
al, 1998). Studies for the identification of prostate cancer genes and other genes related to prostate cancer are on-going, so that the underlying molecular genetic mechanisms contributing to the pathogenesis of the disease will become clearer. Radiation therapy has been successfully applied to both the local and the systemic treatment of prostate cancer. However, tumour responsiveness to radiotherapy can vary between patients. Many studies have demonstrated that this tissue response can reflect the patient’s radiosensitivity \textit{in vivo} and can be related to the radiosensitivity of their cells \textit{in vitro} (Geara et al, 1992; 1992; Alsbeih et al, 1997; Brown et al, 1998; Raaphorst et al, 2000). These studies employ the use of various cell and tissue culture techniques to assess the radiosensitivity of many types of cells and tumours. Radiosensitivity studies of the prostate indicate that significant variability exists within human prostate tumours in the intrinsic radiosensitivity of cells \textit{in vitro} (Leith et al, 1993) and \textit{in vivo} murine models (Thorndyke et al, 1985).

The aim of this study was to assess the radiosensitivity of a number of prostate cancer patients compared to benign prostatic hyperplasia patients by using selected \textit{in vitro} methods and comparing them for their potential use in determining patient intrinsic radiosensitivity and predicting tumor response to radiotherapy. The \textit{in vitro} tests were chosen on the basis of the availability of prostate samples. Whole blood samples and prostate tissue obtained from transurethral resection surgery from various prostate patients with benign prostatic hyperplasia and prostate cancer were the samples obtained for this study. The G2 chromosomal radiosensitivity assay which was standardised in the previous breast cancer study (described in chapter 2), was the first \textit{in vitro} assay to be carried out and whole blood samples of prostate patients were used. The literature demonstrates elevated levels of G2 chromosomal radiosensitivity in a variety of
cancers. Terzoudi et al (2000) demonstrated higher aberration yields in many cancer patients including skin, latynx, brain, bladder and prostate with a mean aberration yield of 2.0 breaks per metaphase in 20 prostate cancer patients compared to control individuals with a mean aberration yield of 1.0 breaks per cell. The objective of this G2 study was to assess the use of the assay for determining radiosensitivity in prostate patients with prostate cancer and BPH, as there are no specific studies in the literature on G2 chromosomal radiosensitivity in prostate patients. A second objective of this study was to extend the investigation and assess if the G2 chromosomal radiosensitivity had any other potential applications to help elucidate some of the underlying cellular mechanism involved in G2 radiosensitivity.

Corresponding tissue samples were obtained from a number of these patients and a primary explant culture technique was carried out. Studies of in vitro primary culture systems to investigate cellular pathways in benign prostatic hyperplasia and prostate cancer have been carried out for almost a century. It was hypothesised that an ideal model system would reproduce the pathology seen in the human disease, demonstrate metastasis, be capable of reproducible replication and sensitivity to hormonal manipulation and also show responses to chemotherapeutic agents and radiation for clinical purposes (reviewed by Merchant et al, 1980).

The use of primary explant culture for the investigation of benign prostatic hyperplasia and prostate cancer dates to the beginnings of the tissue culture method (Burrows et al, 1917), although the first well-controlled studies using the primary explant method were described by Roehl 1958; 1959. Since then, many studies have been carried out to develop a reliable and reproducible in vitro explant model for prostate tissue. However, such attempts have been met with mixed success (Varani et al, 1999; Bayne et al, 1998; Tsugaya et al, 1996; Peehl et al, 1994; 1986; Merchant et
al, 1990; Chaproniere et al, 1986; Weber et al, 1980). In this study, the established primary culture technique from our laboratory (Mothersill et al, 1988; Seymour et al, 1988) was carried out on the prostate tissue samples. This technique has been applied to tissue from a wide variety of organs including; oesophagus, cervix, breast, colon, endometrium, bladder and thyroid. Thus, it was hypothesised that the application of this technique to prostate tissue would yield similar results to those obtained in the previous studies.

Some samples for this study were obtained at QIMR, Brisbane, Australia. This laboratory had expertise in the production of EBV-immortalised lymphoblastoid cell lines from whole blood samples. Infection of B lymphocytes with EBV is a very efficient method for extending their proliferative capacity and making them useful for a wide variety of applications (reviewed by Yeager and Reddel, 1999). They are also ideal for repeat experiments to consolidate scientific data, in contrast to using whole blood cultures from patients which can only be used once per sample. The cell survival assay was carried out on these transformed lymphocytes post-exposure to ionising radiation. The radiosensitivity of these prostate lymphoblastoid cell lines was compared to PC3 and DU145 which are two well known ATCC prostate cell lines (CRL01435 and HTB-81). PC3 was initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62 year old male Caucasian by Kaign et al (1973). DU145 was isolated from Stone at al (1978) from a lesion in the brain of a patient with metastatic carcinoma of the prostate and a 3 year history of lymphocytic leukaemia (ATCC website). Previous studies showed cytotoxic sensitivity to ionising radiation in PC3 cells in contrast to DU145 which also demonstrates the variability of radiosensitivity in prostate cancer patients. Thus, these cells were used as markers of radiosensitivity in the prostate cancer lymphoblastoid cell lines.
Each of these *in vitro* techniques for assessing intrinsic radiosensitivity in prostate patients were compared and contrasted. These techniques are useful biomarkers for predicting intrinsic radiosensitivity in patients and predicting patient tumor sensitivity to radiotherapy.
3.2 MATERIALS AND METHODS

3.2.1 Patients and Samples

Patients with both prostate cancer and benign prostatic hyperplasia were used for this study. They were older men varying in age from 55-85 years. None of the patients had received any cytotoxic chemotherapy or radiotherapy within 6 months of sampling. See table 3.1 and table 3.2 for patient and sample details.

Whole blood samples were taken from prostate patients from two different sources. 13 BPH and 6 prostate cancer blood samples were initially taken by Mr O’Malley, a urological surgeon at St.Vincent’s Hospital, Dublin. The study was extended with the additional 12 prostate cancer blood samples taken by Dr. R. Frank Gardiner, a urological surgeon at Royal Brisbane Hospital, Queensland, Australia. 10ml of whole blood was obtained from patients and placed into sterile universal tubes with heparin sodium salt solution (Sigma) at a concentration of 100IU per ml of blood (St. Vincent’s Hospital, Dublin) Alternatively, 10mls of blood was extracted directly into heparinised vacutainers (RBH, Queensland). The blood samples were kept at ambient temperature prior to culturing and were always used within 24 hours.

The blood samples from patients at St. Vincent’s Hospital were taken during a transurethral resection operation (TURP). Prostate tissue samples (shavings from the TURP) were also taken from these patients and placed into sterile vials with RPMI media (Gibco, Invitrogen Corporation, Carlsbad, CA, USA). Tissue samples were stored at 4°C and were always used within 24 hours.
Table 3.1 Benign prostatic hyperplasia patients’ details. DBPH denotes Dublin BPH patient.

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Age</th>
<th>PSA*</th>
<th>Blood Samples</th>
<th>Tissue Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBPH-1</td>
<td>81</td>
<td>4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-2</td>
<td>78</td>
<td>9.7</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-3</td>
<td>81</td>
<td>7</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-4</td>
<td>87</td>
<td>8.99</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-5</td>
<td>78</td>
<td>1.24</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-6</td>
<td>78</td>
<td>12</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-7</td>
<td>72</td>
<td>1.4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-8</td>
<td>76</td>
<td>3.8</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-9</td>
<td>78</td>
<td>3.48</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-10</td>
<td>90</td>
<td>10</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-11</td>
<td>68</td>
<td>8</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-12</td>
<td>83</td>
<td>8.95</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DBPH-13</td>
<td>78</td>
<td>2.37</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*PSA* - Prostate specific antigen levels
Table 3.2 Prostate cancer patients details. DPC and QPC denote Dublin prostate cancer patients and Queensland prostate cancer patients respectively. NA-Not available.

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age</th>
<th>PSA</th>
<th>Blood Sample</th>
<th>Tissue Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPC-1</td>
<td>82</td>
<td>4.4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DPC-2</td>
<td>79</td>
<td>4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DPC-3</td>
<td>80</td>
<td>NA</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DPC-4</td>
<td>81</td>
<td>8</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DPC-5</td>
<td>74</td>
<td>6.8</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DPC-6</td>
<td>76</td>
<td>6</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>QPC-1</td>
<td>66</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-2</td>
<td>79</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-3</td>
<td>76</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-4</td>
<td>74</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-5</td>
<td>73</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-6</td>
<td>76</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-7</td>
<td>52</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-8</td>
<td>77</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-9</td>
<td>71</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-10</td>
<td>84</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-11</td>
<td>85</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>QPC-12</td>
<td>55</td>
<td>NA</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>
3.2.2 The G2 chromosomal radiosensitivity assay and analysis

The G2 chromosomal radiosensitivity assay was carried out as described in detail in chapter 2. The cytogenetic slides prepared as part of the assay were analysed microscopically. Fifty well-spread and clear metaphases per sample were scored randomly. The number and type of aberrations were recorded per 50 metaphases expressed as a percentage. The bulk of aberrations scored were chromatid gaps and breaks. Terminal deletions, minutes and interarm-exchanges were found less frequently but were included in the G2 score. Endoreduplication and Tetraploidy were scored together in a separate analysis by counting 100 metaphases per slide at x40 microscopical magnification. The radiosensitivity cut-off value of 110 aberrations/metaphases calculated in chapter 2 (from the G2 assay standardisation experiments) was used in this extended study.

Statistical analysis was performed using the Instat program from Graphpad Software Inc (San Diego, CA, USA) calculating the exact significance values (p-value). The Wilcoxin matched pairs test was used to compare non-irradiated versus irradiated G2 scores in all prostate patients. The non-parametric Mann Whitney U-test was used to compare the BPH versus prostate cancer G2 scores. The non-parametric Spearmans correlation test was used to correlate % G2 scores with % tetraploidy/endoreduplication. In each of the prostate patient samples. A significance level of $p \leq 0.05$ was used throughout the statistical analysis.

3.2.3 Prostate tissue culture and subsequent analysis

A. Prostate tissue culture and irradiation

The prostate tissue culture technique used was a slightly altered version of the established explant culture technique of Mothersill et al (1988). Tissue samples (from
13 separate prostate patients (see table 3.1)) were placed onto a sterile petri dish lid for dissection with a small volume of RPMI media. Excess fat and blood was teased off the tissue. The tissue was cut aseptically into small pieces of approximately 0.2 - 0.5mm size and was kept immersed in media to prevent dessication. The tissue fragments were placed in a cocktail of collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.25% trypsin (Gibco) at 1.5mg per ml respectively, and placed into a 37°C incubator for 10 minutes (inverting occasionally to mix) to digest the tissue. The digested tissue fragments were individually plated into T24 flasks (Nunc, Invitrogen) in 2mls of RPMI 1640 media (Sigma) supplemented with 12.5% of foetal calf serum (Gibco), 7.5% of horse serum (Gibco), 10mM L-glutamine (Gibco), 20IU penicillin and 20 μg streptomycin (Gibco), 1mg/ml Hydrocortizone (Sigma) and 100mIU/ml insulin (Sigma). The cultures were incubated at 37°C, 5% CO₂ for 5-7 days for attachment and initial epithelial proliferation. The explant cultures were examined for attachment and initial epithelial outgrowth from the tissue. The cultures were irradiated with various low doses of gamma radiation (0-5Gy) on a cobalt-60 teletherapy unit (St. Luke’s Hospital, Rathgar) and were then re-incubated at 37°C at 5% CO₂ for a further 10-14 days to grow. All explant cultures were then fixed with 5ml of 10% neutral buffered formalin (see appendices) and left at room temperature for subsequent immunocytochemical analysis.

**B. Immunocytochemical analysis of explant cultures**

Immunocytochemical staining was carried out using the strepavidin peroxidase method using a Vectastain ABC mouse monoclonal kit (Vector laboratories Incorporated, Burlingame, CA, USA) as the antibodies used in this study were all monoclonal. A negative control was always included in each immunocytochemical analysis.
staining experiment. The flasks were cracked open exposing the explant culture. They were circled with a Dako hydrophobic pen (Dako, Glostrup, Denmark) to ensure that there was no loss of reagents from the explant culture. All procedures were carried out at room temperature. The explant cultures were rehydrated in phosphate buffered saline (PBS) (See appendices). 3% of hydrogen peroxide (Sigma) was added to the explants for 5 minutes, to block endogenous peroxidase activity before staining.

The explants were incubated with normal blocking serum provided by the kit for 20 minutes. The primary antibody was diluted appropriately and applied to the cultures for 30-60 minutes. Various primary antibodies were applied to the cultures. These included; cMyc (Novocastra, Newcastle Upon Tyne, UK), Bel2 (Dako) and P53 (Novocastra) at various optimal concentrations recommended on the supplied data sheets. The biotinylated secondary antibody supplied by the kit was then added to the explant cultures and incubated for 30 minutes. The slides were washed three times in PBS and the strepavidin peroxidase reagent (ABC reagent) supplied by the kit was applied to the cultures for 30 minutes. The explant cultures were then incubated with diaminobenzidine (DAB) (Sigma) for 10 minutes in darkness. The cultures were washed well in water to remove excess chromagen and they were counterstained with harris haematoxylin (BDH) for 1 minute, washed thoroughly and mounted with glycergel (see appendices) and a coverslip and dried overnight at room temperature.

The explant cultures were analysed using a Leica microscopical system. Blue cellular staining indicated negative expression of the antibody whereas brown staining indicated positive expression of the antibody.
3.2.4 Radiation sensitivity of cells from prostate patients

A. EBV-immortalisation of lymphoblastoid cell lines

5mls of whole blood was retained from prostate cancer patients QPC1-QPC12 to isolate the lymphocytes and transform them with EBV virus and produce an immortalized lymphoblastoid cell line for subsequent experimental use. This included the following procedures; 5mls of whole blood was diluted in 5mls of sterile PBS (See appendices) and mixed by gentle inversions. 7mls was carefully layered over 3mls of ficoll paque (Sigma) in a sterile 10ml centrifuge tube and it was centrifuged at 2000rpm for 20 minutes at room temperature to separate the blood cells. The lymphocytes were carefully collected from the interphase layer and transferred to a new sterile 10ml tube. The lymphocytes were washed twice in 5mls of sterile PBS through centrifugation at 1000rpm for 10 minutes. The supernatant was removed leaving approximately 1ml of PBS over the cell pellet for resuspension. 250μl EBV virus (made from B958 cells at QIMR, Brisbane, Australia) was added to the lymphocytes and incubated overnight at 37°C to allow sufficient transformation of the lymphocytes with EBV virus. 4mls of RPMI-1640 media (supplemented with 10% foetal calf serum (Gibco) and 2μl/ml of a 10mg/ml stock solution of phytohaemagglutinin (PHA-P) (Miurex)) was added to the lymphocytes and they were serially diluted and plated out into a 24-well plate (Nunc). The plates were incubated at 37°C with 5% CO₂ for 4 weeks changing the media once per week. The cells were then transferred into flasks with culture media to increase the volume of cells and media.
**B. Sensitivity of prostate cells to ionising radiation**

The cell survival assay was used to measure sensitivity of the prostate cancer lymphoblastoid cell lines (LCLs) versus control LCLs to low doses of ionising radiation. The prostate adherent cells; PC3 and DU145 versus control HPV-G (Human papilloma virus) cells were also used. The prostate adherent cells; PC3 and DU145 were grown to approximately 80% confluency. The cells were digested with equal volumes of 0.25% trypsin (Gibco) and 1:1000 versene (Gibco) at 37°C for 5-10 minutes (checking microscopically on occasions) to remove them from the bottom of the flask. The trypsinised cells were counted on a Coulter particle counter (Z1 model, ETL laboratories, Cortland, NY, USA) by adding 1 ml of cell suspension to 40mls of Isoton II (Beckman Coulter, Krefeld, Germany) which is an azide-free electrolyte solution specifically used with the instrument. The cell number per ml of cells was calculated (by multiplying the reading on the counter by 42 to allow for the 1 ml of cells in 20 mls of isoton and a 0.5 aperture reading) and a known number of cells was plated into each flask (in triplicate) with DMEM F-12 culture media (supplemented with 10% foetal calf serum (Gibco), 10mM L-glutamine (Gibco), 20IU/ml of penicillin (Gibco) and 20µg/ml of streptomycin (Gibco). The cells were incubated overnight at 37°C in a 5%CO₂ environment. The cells were irradiated with 0.5Gy and 1Gy of gamma radiation, and the control cells were sham-irradiated. The cells were re-incubated at 37°C for 10-14 days to allow colony formation. The cells were stained with 1:5 carbol fuschin (BDH) in tap water for 5 minutes and washed well. The colonies were counted for each dose per triplicate flask and the % cell survival calculated from the initial cell numbers plated. The calculation was as follows:
% Survival = \frac{\text{number of colonies counted} \times \text{Survival fraction of 0(Gy) dose}}{\text{number of cells initially plated}} \times 100

Lymphoblastoid cell lines (which are suspension cultures) were mixed well by pipetting to break up cellular clumps. All prostate cells were counted with a coulter counter (ETL laboratories) as described above and plated at \(2 \times 10^5\) per ml per flask set up in triplicate per dose point. The cells were irradiated with 0.5 and 1Gy of gamma radiation. The cells were placed back into the incubator for 48 hours. 1ml of cells was suspended in 20mls of isoton solution (Beckman coulter) and counted on the coulter counter. The cell number per ml was calculated by multiplying by 42 (described above) and the % cell survival was calculated. The calculation was as follows:

% Survival = \frac{\text{number of cells counted} \times \text{survival fraction of 0.5Gy dose}}{\text{number of cells initially plated}} \times 100

Storage and maintenance of all cell lines

Adherent cell lines and lymphoblastoid cell lines were stored in liquid nitrogen. See appendices for freezing down and resuscitation of cells.
3.3 RESULTS

3.3.1 G2 radiosensitivity scores of prostate patients (PC and BPH).

Radiosensitivity was determined by the induction of chromatid aberrations in the G2 phase of the cell cycle in response to radiation exposure (Sanford et al, 1989). The radiation induced chromatid breaks and gaps were calculated by subtracting the spontaneous aberrations yields from those recorded in the irradiated samples. See chapter 2 for pictures of aberrations induced by ionising radiation. A G2 score was calculated by multiplying the aberration yield by 100 and those scores exceeding the cut-off value of 110 aberrations per 100 metaphases (1.10 aberrations per metaphase) were recorded as G2 radiosensitive. This was calculated in all of the samples in the control, prostate cancer and benign prostatic hyperplasia groups. Table 3.3 shows the radiation induced aberration yields per metaphase in each of these groups and the number of donors who were recorded as G2 radiosensitive per group. Figure 3.1 and 3.2 demonstrates the differences in G2 scores between the patients with prostate cancer and BPH.

The Mann-Whitney U-test was carried out to compare the control group with each of the prostate patient groups. The difference between the controls (0.87 ± 0.15) and the patients with benign prostatic hyperplasia (1.24 ± 0.49) was significant (p < 0.08) and the difference between the controls (0.87 ± 0.15) and the prostate cancer patients (1.50 ± 0.30) was considered extremely significant (p < 0.001). Each of the prostate groups was also compared using the Mann-Whitney U-test and the difference between the benign prostatic hyperplasia and prostate cancer group was just short of significance (p < 0.07). Using the G2 radiosensitive cut-off value, 5/13 of the patients
with BPH were recorded as G2 radiosensitive compared to 13.18 prostate cancer patients who were determined to be G2 radiosensitive.

3.2 Endoreduplication and Tetraploidy
Defective repair of DNA damage is associated with elevated chromosome aberrations including endoreduplication and tetraploidy (Halappanavar and Shah, 2004; Yoshihara et al, 2004). Endoreduplication and tetraploidy were scored in a separate metaphase analysis due to their distinct characteristic features. Endoreduplication was characterised by the appearance of identical parallel copies of each chromosome which are formed by replication and without separation lie side by side as thick cable like structures (figure 3.4). Tetraploidy was characterised by the appearance of duplicate copies of each chromosome which are formed by replication and separate within an intact nuclear membrane which does not divide and this is demonstrated in figure 3.5. Double the normal number of chromosomes, 92, was observed in both processes.

3.3 Correlation of G2 radiosensitivity with endoreduplication and tetraploidy
Radiation induced G2 radiosensitivity scores were correlated with radiation induced % endoreduplication and tetraploidy (% Endo/Tetra) in each prostate group using the Spearmans rank correlation test. The correlation between % Endo/tetra and G2 radiosensitivity in BPH patients was not significant (p > 0.2660) and this is illustrated in figure 3.6. In contrast the correlation of % Endo/Tetra and G2 radiosensitivity in prostate cancer patients was significant (p ≥ 0.0007) and this is illustrated in figure 3.7 with a trendline added to the scatterplot to show this inverse correlation with an increase in G2 scores and a decrease in % Endo/Tetra.
Table 3.3 G2 radiosensitivity in the control donor group compared to the prostate patient groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Donors</th>
<th>Mean*± SD</th>
<th>Range*</th>
<th>No. of G2 radiosensitive</th>
<th>Difference (p)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>0.87 ± 0.15</td>
<td>0.68 - 1.15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BPH</td>
<td>13</td>
<td>1.24 ± 0.49</td>
<td>0.72 - 2.12</td>
<td>5</td>
<td>P &lt; 0.08</td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>18</td>
<td>1.50 ± 0.30</td>
<td>1.06 - 1.94</td>
<td>13</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

* Range of aberrations

** Number of donors exceeding the G2 radiosensitivity cut-off value (1.15 aberrations per metaphase).

*** Difference of the control group compared to the prostate groups.
Figure 3.1 Histogram illustrating G2 scores of BPH patients and the radiosensitivity cut-off point.

![Histogram illustrating G2 scores of BPH patients and the radiosensitivity cut-off point.](image1)

Figure 3.2 Histogram illustrating G2 scores of prostate cancer patients and radiosensitivity cut-off point.

![Histogram illustrating G2 scores of prostate cancer patients and the radiosensitivity cut-off point.](image2)
Figure 3.3 Micrograph of a control (non-irradiated) metaphase spread. 46 fully intact chromosomes were observed.

Figure 3.4 Micrograph of endoreduplication. Identical parallel copies of each chromosome were seen lying side by side like thick cable-like chromosomes.
**Figure 3.5** Micrograph of tetraploidy. Double the number of chromosomes with the same karyotype was observed (92) as with endoreduplication, however each identical chromosome was separated within the metaphase.
Figure 3.6 Scatterplot showing no correlation pattern between the G2 radiosensitivity scores and % Endo/Tetra in BPH patients.

![Scatterplot of BPH G2 scores and % Endo/tetra](image)

Figure 3.7 Scatterplot showing a distinct correlation (with the additional trendline) between the G2 scores and % Endo/tetra in prostate cancer patients.

![Scatterplot of prostate cancer G2 scores and % endo/tetra](image)
3.3.2 Immunocytochemical analysis of explant cultures

The objective of carrying out immunocytochemical analysis on the explanted prostate tissue was to show expression of proteins in epithelial cells which grew from irradiated explants and to examine the cultures for qualitative evidence of apoptosis after irradiation and to subsequently attempt to carry out a quantitative analysis of protein expression and apoptosis on these prostate cultures.

The attachment efficiency of the prostate explant cultures was very low. It varied between 5-25%. Thus the immunocytochemical analysis was limited. The explanted cells that were stained were epithelial. Epithelial cells were described as irregular shaped cells which grow together to form sheets or layers (reviewed by Leeson at al, 1973). Figure 3.8 shows a negative control explanted sample. The cells were morphologically described as epithelial. Blue staining indicates negativity while brown staining indicates positivity in immunocytochemically stained samples. The figures demonstrated in this study were derived from the immunocytochemical analysis of the prostate cancer explanted samples. Figure 3.9 demonstrates positive expression of cMyc in the prostate cancer cells observed in the cytoplasm of the prostate epithelial cells. Figure 3.10 demonstrates both positive and negative expression of Bcl2 in the cytoplasm of the prostate cancer cells. The brown positive staining was more discreet in Bcl2 than in cMyc, where the intensity of the brown colour is weaker. Figure 3.11 demonstrates both positive and negative expression of P53 in both the nucleus and cytoplasm of the prostate cancer cells. The positive staining with P53 was similar to the Bcl2 staining.
**Figure 3.8** Negative Control: Explant culture showing epithelial cells with blue (negative) immunocytochemical staining in the nucleus and cytoplasm.

![Typical Epithelial cells]

**Figure 3.9** Prostate cancer explanted sample immuncytochemically stained for cMyc expression. Brown colour observed in the cytoplasm of the cells indicates positive expression of cMyc in these cells.

![Stronger intensity of cMyc expression]

![Positive expression of cMyc but intermediated compared to other areas]
**Figure 3.10** Prostate cancer explanted sample immunocytochemically stained for Bcl2 expression. Brown staining in the cytoplasm indicates positive expression in some cells and blue staining indicates negative expression in other cells present.

**Figure 3.11** Prostate cancer explanted sample immunocytochemically stained for P53 expression. Brown staining indicates positive expression in contrast to blue staining which indicates negative expression. Nuclear and cytoplasmic expression of P53 can be seen.
3.3.3 Radiation sensitivity of prostate cancer cell lines

The radiation sensitivity of all cells was calculated from the % cell survival of the cells following exposure to ionising radiation. The cells were exposed to low doses of radiation only; 0.5 and 1 Gy. Figure 3.12 demonstrates the cell survival of PC3 and DU145 prostate cell lines in contrast to the HPV-G control cell line. PC3 and DU145 were extremely different in radiosensitivity with PC3 being more sensitive. DU145 were more radioresistant than the HPV-G control cell line. The degree of radiosensitivity between these cell lines was used as a marker for radiosensitivity in the lymphoblastoid cell lines (LCLs) made from blood samples from prostate cancer patients (QPC) because both of these cell lines represented prostate cells that showed radiosensitivity at each end of the spectrum. The PC3 demonstrated hypersensitivity to ionising radiation in contrast to DU145 cells which demonstrated radioresistance to ionising radiation. Figure 3.13 demonstrates the radiosensitivity of three selected prostate cancer LCLs chosen on the basis of their radiosensitive G2 score, in contrast to a control LCL (C2ABR). Sample code QP-5 and QP-9 showed the same levels of radiosensitivity as PC3 in figure 3.12. These samples also had high G2 radiosensitivity scores. Sample code QP-2 had a low G2 score and showed similar radiosensitivity to the control (C2ABR). The radiosensitivity of QP-2 and C2ABR were similar to the HPV-G control of figure 3.12. None of the samples showed radioresistance similar to DU145. The G2 scores of the patients coded QPC-2, QPC-5 and QPC-9 (cytogenetic radiosensitivity at 0.5Gy) was plotted against their % survival value at 0.5Gy (Figure 3.14). High G2 scores correlated with low % survival values in QPC-2 and QPC-5. In contrast, low G2 scores correlated with high % survival in QPC-9 demonstrating that these cells were not radiosensitive. This
correlation indicates that both methods are useful for detecting radiosensitivity in patients' samples.

**Figure 3.12** Radiation sensitivity of prostate (PC3 and DU 145) versus control (HPV-G) cell lines
**Figure 3.13** Radiation sensitivity of selected lymphoblastoid cell lines (made from prostate cancer patients’ blood samples) compared to a control LCL.

![Graph showing radiation sensitivity of selected lymphoblastoid cell lines](image)

**Figure 3.14** Correlation of G2 scores with % survival from two *in vitro* methods for radiosensitivity using prostate cancer patients (QPC-2, QPC-5 and QPC 9) lymphocytes.

![Graph showing correlation of G2 scores with % survival](image)
3.4 DISCUSSION

The underlying molecular mechanisms associated with prostate cancer formation and progression has yet to be elucidated. Also radiotherapy as a treatment gives highly variable results.

The biological characterisation of individual cells and tumours may ultimately provide strategies to individualise cancer therapy. One such aspect of this characterisation is the assessment of radiosensitivity in patient cells. This has led to the development of many biological assays that can be applied to a patient’s cells or tissue with potential as rapid tests for predicting radiosensitivity. Chromosomal radiosensitivity is a potential endpoint for assessing the intrinsic radiosensitivity within patients. The G2 chromosomal radiosensitivity assay which was standardised in chapter 2 was applied to whole blood samples from all prostate patients with benign prostatic hyperplasia and prostate cancer. The radiosensitivity cut-off value (110 aberrations per metaphases) calculated in chapter 2 from the intra and inter-control data was used as the cut-off value in this prostate study. Using this radiosensitivity cut-off value, it was calculated that 5/13 benign prostatic hyperplasia samples were G2 radiosensitive in contrast to 13/18 prostate cancer samples which were G2 radiosensitive. Furthermore, the difference between G2 radiosensitivity of prostate cancer patients and BPH patients was considered very significant with a p-value of 0.0060. This indicates that there are different underlying mechanisms contributing to G2 radiosensitivity in BPH and prostate cancer patients.

Radiosensitivity in cells has been related to double strand break repair (reviewed by Nyberg et al, 2002). The identification of a radiosensitive sub-population in males with prostate cancer could associate the genes and mechanisms of DSBR as a possible pathway in prostate cancer formation. Prostate cells communicate through signalling
mechanisms employing the use of growth factors. Growth factors are essential for prostate homeostasis (proliferation/differentiation) and are often controlled by androgens. The growth factor network can be disrupted through cellular damage and other factors, which can include loss of androgen control. It is possible that this mechanism may be involved in the increased G2 radiosensitivity of BPH samples. Further studies to investigate the underlying mechanisms, associated genes and protein products involved in G2 radiosensitivity of the prostate are required. An observation that was made while microscopically analysing the slides from the G2 chromosomal radiosensitivity assay (of the initial patients (DPC and DBPH)) was that endoreduplication and tetraploidy were present at higher levels in some samples than others. Thus, endoreduplication and tetraploidy were recorded separately and matched with the corresponding G2 score of the patients. Percentage endoreduplication and tetraploidy (%Endo-Tetra) were present at higher levels in the prostate cancer patient’s and a correlation between the G2 scores and the % Endo-Tetra was considered significant with a p-value of $\geq 0.0007$. The % Endo-Tetra levels in the BPH patients varied and were lower than this value and the correlation between the G2 scores and the % Endo-Tetra was considered not significant with a p-value of 0.2660. Endoreduplication and tetraploidy were classed together as they are both processes that occur in cells that have lost the power to divide mitotically, usually forming due to a spindle defect.

In the present study we demonstrate that there is a distinct inverse correlation between G2 radiosensitivity the spindle defects, endoreduplication and tetraploidy (%Endo/Tetra) which are indicative of specific cell death mechanisms. The highest G2 radiosensitivity scores had the lowest % Endo/Tetra, and the lowest G2 radiosensitivity scores had the highest % Endo/Tetra. From this data, we
hypothesised that all cells exposed to 0.5Gy of ionising radiation are halted at G2 checkpoint as the first defense mechanism to the genotoxic insult, and the fate of the cells are decided at this point. Damaged radiosensitive cells undergo apoptosis while radioresistant damaged cells can surpass this checkpoint and progress in the cell cycle to a post-mitotic stage leading to the initiation of mitotic errors such as endoreduplication and tetraploidy in specific endocycles and subsequently delayed reproductive death or mitotic death. See section 1.7 of chapter 1 for more details on mitotic death. Many cell cycle checkpoints operate in response to DNA damage from ionising radiation and can initiate these cell death mechanisms. A group of checkpoints are involved in monitoring cytoskeletal function and thus the formation of endoreduplication and tetraploidy. These include a retinoblastoma pocket protein-dependent G1 checkpoint that monitors actin function (Lohez et al, 2003), a P53-dependent G1 checkpoint that monitors microtubule function (Trielli et al, 1996), a G2 checkpoint sensitive to micro-tubule function (Rieder et al, 2000) and a spindle assembly checkpoint which monitors microtubule polymerization for the attachment of chromosomes at each polar end and tension of the spindle to ensure segregation of the chromatids in anaphase (Soufias et al, 2001). These G2 and spindle assembly checkpoints function in response to DNA damage for maintaining genomic integrity (reviewed by Andreassen et al, 2003). Thus, the correlation between G2 radiosensitivity and these spindle defects (endoreduplication and tetraploidy) in prostate cancer patients, elucidates some of the underlying mechanisms involved in the response to DNA damage from ionising radiation exposure and strengthens the hypothesis that apoptosis and mitotic death and the main death mechanisms that result from low dose radiation exposure and that both processes are equally important cell death mechanisms induced by IR.
Attempts to elucidate the genes and protein products that may be involved in radiosensitivity of prostate patients were made by applying a well established primary culture technique to prostate tissue samples from corresponding G2 scored blood samples and analysing the expression of various oncogenes and tumor suppressor genes post-irradiation through immunocytochemistry. 13 prostate tissue samples were received with every blood sample taken from St. Vincents hospital (See table 3.1) and between 20-60 flasks (depending on the available tissue) were set up for each of the patient samples. The established tissue culture technique from our laboratory (Mothersill et al, 1988; Seymour et al, 1988) was applied to these corresponding tissue culture samples. This explant culture technique was successful with normal tissue derived from a wide variety of organs including; oesophagus, cervix, breast, colon, endometrium, bladder and thyroid. Therefore, it was postulated that it could be successful for culturing both normal and malignant prostate tissue. However, much difficulty was experienced using this technique on prostate tissue. The attachment efficiency varied from 5%-25% with the majority at the lower end of the spectrum and not all of the cultures were of epithelial origin. The lowest attachment efficiencies were observed in prostate cancer tissue samples, and these proved more difficult to culture. The problems experienced could be due to many factors; Prostate tissue is made up of a heterogeneous cell population including stromal cells (mesenchyme) which comprises of smooth muscle cells and fibroblasts and epithelial cells from basal or luminal (secretory cells) origin. Thus separation of these cells can be more difficult depending on the content of the various cells received in the sample. The desired cell type was epithelial from basal origin. A study by Varani et al (1999) was carried out demonstrating the characteristics of malignant and non-malignant prostate tissue organ culture. They demonstrated that in non-malignant prostate tissue
8 days in culture, the secretory/luminal epithelium was lost whereas basal epithelial cells were increased to fill the lumen of the gland and the basement membrane remained intact. In contrast malignant tissues that were anaplastic to begin with remained so and no proliferation was observed, and differentiated tumours continued to express features of differentiation with a thin and discontinuous basement membrane observed in places. They concluded that both tissues could be maintained in primary organ culture but non-malignant tissue had greater proliferative capacity. This appeared to be the case with the prostate cancer samples which were more difficult to culture than BPH tissue samples. Many earlier studies on prostate explant cultures was carried out by Merchant (1980, 1983, 1987). He postulated that an explant of human prostate tissue containing viable acini will give rise \textit{in vitro} to an outgrowth of epithelial cells of presumed basal origin and thus all failure to obtain growth are due to the absence of viable acini in the tissue specimen (Merchant 1990). It was suggested that the choice of cauterizing electric current by the surgeon in transurethral resection examination (TURP) was an important variable associated with prostate tissue culture. Lack of attention of this variable will result in acinar tissue shown by pathologic examination to contain damaged acini. Thus, in obtaining TURP specimens, the electric current should be reduced to eliminate heat damage (Merchant, 1990). All of the prostate tissue samples obtained were taken by Mr. Kiaran O’Malley at St. Vincents Hospital, Dublin and couriered accordingly. We did not have any input into the clinical procedures involved in obtaining the samples and thus the electrical current used for the TURP procedure was unknown. However, this is an important consideration for future experimentation. Further factors could also have contributed to low yield of prostate explant cultures. For example, from previous tissue culture experience with urothelium, there appeared to be a trend that
tissue derived from older patients did not grow in culture as well as those derived from younger sources. Finally, the irradiation procedure in the tissue culture protocol may also be a factor contributing to the low yield of explants. The cultures had to be transported to a cobalt-60 teletherapy unit and the explants became dissociated from the flasks on occasion. An important consideration of this technique for future application is to positively identify that the prostate tumour tissue used for explanting is indeed prostate tumour. In the TURP examination, shavings of the prostate are taken from areas which are presumably the prostate tumour. The use of prostate tumour markers such as AMACR or CK18 could be used to distinguish between benign and tumour prostate tissue for subsequent explantation.

However, immunocytochemical analysis was carried out on all available prostate explant samples. One set of explants from a prostate cancer patient (DPC-4) which was also G2 radiosensitive were of interest for this study. These explant samples were stained for the expression of cMyc, Bcl2 and P53 cell cycle proteins. Strong staining for cMyc (figure 3.7) was observed in contrast to P53 and Bcl2 staining. Positivity was observed in both P53 and Bcl2 but to a lesser extent with many negative cells also present (figure 3.8 and 3.9). Previous studies have shown expression of these oncogenes in malignant and non-malignant prostate tissue. cMyc expression in prostate tissue was shown by Fleming et al (1998) and Matusik et al (1987). Bcl2 expression in the prostate was shown by McDonnell et al (1992) and P53 expression in prostate tissue was demonstrated by many groups (Visakorpi et al, 1992; Bookstein et al, 1993; Aprikian et al, 1994) and others which were reviewed by Prendergast and Walther (1995). cMyc and Bcl2 are nuclear transcription factor oncogenes which are found in many tumours and they are involved in cellular proliferation. P53 is a tumour suppressor gene which suppresses tumor formation by
triggering cell cycle arrest to allow repair processes to occur or trigger apoptosis (See section 1.7, chapter 1 for a detailed description). The limited immunocytochemical results demonstrated in this study were consistent with the hypothesis described by Mothersill et al (1997), that strong staining for cMyc coupled with low or absent Bcl2 expression generally correlated with radiosensitivity. The results in this study which showed this were also obtained from a G2 radiosensitive prostate cancer patient. However, due to the limited availability of samples this could not be repeated.

The prostate explant tissue culture technique was unsuccessful for this study but the limited results obtained were promising. The method could be improved for further use with alteration of the variables that exist clinically and experimentally and may be a potentially useful method for predicting patient tumour sensitivity.

The final in vitro technique used for the assessment of radiosensitivity within prostate patient samples was the production of EBV-immortalised lymphoblastoid cell lines, exposure to ionising radiation and radiosensitivity estimated from the cell survival results. This was carried out on the QPC samples only as the expertise and access to the EBV virus was in the QIMR laboratory, Brisbane, Australia. The G2 assay was also carried out on the whole blood samples from these patients and so the radiosensitive samples had been identified. Thus, the cell survival assay was not carried out on all of the LCLs made. Two patients LCLs with very high G2 scores and one patient LCL with a normal G2 score were chosen to compare the G2 radiosensitivity scores with cell survival post radiation exposure. The radiation dose carried out for the G2 assay was 0.5Gy, therefore the radiation doses used for the cell survival assay was 0.5Gy and 1Gy. The cell survival of PC3 and DU145 adherent cell lines (exposed to the same radiation doses) were also plotted and compared to the cell survival of these LCLs. The cell survival of the LCLs correlated with the G2
radiosensitivity scores showing the two patient cells with high G2 scores to be the most sensitive with lower % cell survival. These were similar to the values obtained with PC3, which acted as a prostate radiosensitive marker.

Each of the in vitro techniques used for assessing intrinsic radiosensitivity had advantages and disadvantages. The prostate explant technique had the most disadvantages in this instance but has the potential to be a very useful diagnostic biomarker for assessing radiosensitivity through the expression of various proteins in BPH and prostate cancer. However, this requires further work and access to an abundant source of prostate tissue. The G2 assay has proved to be a reliable in vitro test for predicting radiosensitivity in patient samples, observed in both breast cancer (chapter 2) and patients with BPH and prostate cancer. Blood samples are obtained more easily than tissue samples. Complications associated with this assay are that the lymphocyte cultures are cultured in medium with no antibiotics and are therefore susceptible to cell culture contamination from bacteria and fungus. In cases like this, a repeat sample must be taken from the patient for the analysis and this was not an option in this study.

The use of lymphoblastoid cell lines are beneficial for this reason. The proliferative capacities of the lymphocytes are extended and repeat experiments can be carried out. The G2 assay was carried out on some of these LCLs and the G2 score was similar to that from the G2 assay using whole blood cultures. However, standardisation of the G2 assay for use with LCLs would need to be carried out for further use. The disadvantage of this system is that it is too time-consuming for routine clinical application.

In conclusion, out of the three in-vitro cell culture methods used for assessing the radiosensitivity of a patient sample, the G2 assay was more suitable for routine
clinical application than the other two methods due to availability of samples and time for analysis. For experimental purposes, the use of lymphoblastoid cell lines would be preferred due to the abundance of cells.
CHAPTER 4

THE RELATIONSHIP BETWEEN ATAXIA-TELANGECTASIA GENE CARRIERS AND CANCER PREDISPOSITION

4.1 Introduction
4.2 Materials and Methods
4.3 Results
4.4 Discussion
4.1 INTRODUCTION

The autosomal recessive disease ataxia telangiectasia is a disease characterised by the clinical features of progressive cerebellar ataxia, oculocutaneous telangiectasias and immunodeficiency and the cellular features of genomic instability, cancer predisposition and hypersensitivity to ionising radiation (Gattì, 2000).

The causative gene which was identified by Savitisky et al (1995) is known as the ataxia-telangiectasia mutated (ATM) gene. This gene encodes a member of the phosphatidylinositol-3 kinase (PIK) family of proteins. In A-T patients, mutations in the ATM gene have been linked to cancer phenotypes, primarily leukemias and lymphomas (Olsen et al, 2001). Heterozygous carriers of mutations in ATM do not have the A-T clinical phenotype but have a higher cancer predisposition.

It was estimated that about 1.4% of the general population are heterozygous carriers for ATM (Swift et al, 1986). These heterozygotes have been linked to predisposition to cancer. Swift et al (1991) conducted an epidemiological study on 161 families affected with Ataxia-telangiectasia. He recorded all types of cancers occurring such as breast, prostate, colon, lung, pancreas, stomach etc and he estimated an increased relative risk of cancer in A-T heterozygotes in both men and women with a particular emphasis on breast cancer risk in women.

Murine models were employed to demonstrate that mouse carriers of mutated ATM that are capable of expressing ATM, have an increased susceptibility to cancer (Spring et al, 2002). This was demonstrated in different tumour types such as sarcomas, lymphomas, adenomas, ovarian tumours, leukemias and dermoid cysts.

A small proportion of prostate cancer radiotherapy patients who experience severe late effects were shown to be A-T heterozygotes (Hall et al, 1998). Furthermore,
heterozygous carriers of the ATM gene have been suggested to be indirectly involved in HNPCC (Human non-polyposis colon cancer) predisposition through modulating the penetrance of MLH1 and MLH2 germ-line mutations (Maillet et al, 2000). However, the contribution of A-T heterozygosity predisposing to these various cancers is estimated to be very small.

ATM has been primarily linked to breast cancer predisposition through epidemiological studies (Swift et al, 1987; 1991; Pippard et al, 1988; Borreson et al, 1990). These studies were analysed by Easton (1994) who estimated that A-T heterozygotes would account for between 1 – 13% of breast cancer cases, with 3.8% being the best estimate. Studies which followed this showed similar risk estimates (Athma et al, 1996; Inskip et al, 1999 and Janin et al, 1999) with the exception of the study by Stankovic et al (1998) which showed an elevated risk estimate of 12.7%.

There is a diverse spectrum of ATM mutations found in A-T patients. These mutations are derived from two different classes; truncating ATM mutations which are nonsense, frame-shift or splicing mutations that truncate the protein and missense mutations from small in-frame insertions/deletions. It was estimated that approximately 70-90% of mutations identified in A-T patients are truncating mutations (Stankovic et al, 1998; Concannon and Gatti, 1997). However, this figure is thought to be overestimated due to the bias of the protein truncating test (PTT) which was used to screen for A-T truncating mutations (Fernet et al, 2004). These can easily be detected due to the unstable protein that is present in the cell. In recent years, there has been a shift in detecting A-T mutations from protein truncating mutations to missense mutations. Missense mutations lead to the expression of a more stable mutant protein and recent studies demonstrate these mutations in breast cancer patients (Scott et al, 2002; Sommer et al, 2002; 2003).
Although missense mutations account for the minority of mutations found in A-T compared to truncating mutations, it was hypothesised that these mutations in the ATM gene are the most important class of mutations predisposing to breast cancer (Sommer et al, 2002).

Three specific missense mutations were used for this study. These were T7271G and IVS10-6T→G and 7636del9 ATM mutations found on exon 51, 11 and 54 respectively.

Stankovic et al (1998) found that the ATM missense mutation T7271 elevated the risk of breast cancer 13-fold in both heterozygotes and homozygotes in a study conducted on families in the British Isles. Broeks et al (2000) conducted a study on Dutch breast cancer patients and they described the IVS10-6T→G missense mutation to increase the risk of breast cancer by 9-fold. Several studies were carried out on both of these ATM mutations to estimate breast cancer risk in breast cancer families that were non BRCA1/2 carriers (Chenevix-Trench, 2002) and in patients with unilateral and bilateral breast cancer (Bernstein et al, 2003). However, in these two studies the prevalence of these ATM mutations for breast cancer predisposition was low and it was thought that this may reflect population heterogeneity. Thus, the ATM mutations T7271G and IVS10-6T→G may predominate in European populations and were ideal candidates for screening the Irish breast cancer patients in this study which were also non BRCA1/2 carriers.

The 7636del9 mutation represents one of the most common mutations found in A-T patients. A murine model (Δ SRI mouse) was set up by Spring et al (2001) where this nine-nucleotide in frame deletion was introduced into the ATM gene by homologous recombination. This deletion mutation results in the loss of three amino acid residues but produces near full-length ATM protein and lacks kinase activity. Furthermore the
observed phenotype was different, reflecting heterogeneity seen in A-T patients with
different mutations. This mutation was also a candidate for screening the breast
cancer samples in this study.

Denaturing high-performance liquid chromatography (dHPLC) is a highly sensitive
method for detecting single nucleotide polymorphisms (SNPs), mutations and other
DNA sequence variants by fractionation of homo- and heteroduplex DNA on reverse-
phase chromatography columns under partial denaturation.

dHPLC has been shown to be a superior method to conventional methods for mutation
detection in blind analysis studies (O’Donovan et al, 1998). Near 100% specificity
has been shown in many studies reviewed by Xiao and Oefner (2001) and they
concluded that the sensitivity and specificity of dHPLC appears to be consistently
higher than 96%.

Because of its high specificity for mutation detection analysis, it has been used in
previous studies for detecting ATM mutations in breast cancer cases (Szabo et al,

dHPLC operates by comparing two or more chromosomes as a mixture of denatured
and reannealed PCR products revealing the presence of the sequence variant or SNP
by partial denaturation of homoduplex and heteroduplex DNA. Homoduplexes are
formed from homozygous DNA and heteroduplexes are formed from heterozygous
DNA. These both have different melting properties and dHPLC functions by
exploiting these properties for the detection of mutations (Oefner and Underhill, 1995;
1998; 1999). The detection of sequence variants for the positional cloning of the
genes is only the first step in this process. Actual mapping of the gene requires
genotyping those variants. Many methods exist for genotyping. One of these
methods is the genotyping of SNPs by primer extension and HPLC (Hoogendoorn et
al, 1999) which is then complete dHPLC. This enables direct genotyping of biallelic sites contained in short amplicons as well as analysis of primer extension products. Thus, accurate measurement of allele frequencies in pooled samples of DNA using primer extension and dHPLC offers an efficient and economical way to genotype SNPs in large case-control and family-based association studies (Xiao and Oefner, 2001).

This study involved the screening of a population of breast cancer patients’ samples using the complete dHPLC technique described above, for the specific T7271G, IVS10-6T→G and 7636del9 A-T mutations.
4.2 MATERIALS AND METHODS

4.2.1 Patients

DNA samples from 57 breast cancer patients (10/20μl volume) were obtained from the National Centre for Medical Genetics, Dublin. These samples were all coded but were known non-BRCA1/2 carriers (sporadic breast cancers). The samples were personally transported to the Queensland Institute of Medical Research (QIMR), Brisbane where this study was conducted.

4.2.2. PCR amplification

Each sample was quantified separately in 1/100 dilution in TE (Tris-EDTA) buffer at a wavelength of 260nm. With the known concentration, a working solution of each sample could be made for subsequent PCR. The DNA working solution was 20ng/μl DNA concentration made up in 10mM Tris solution at PH 8 to a 25μl volume.

Forward and reverse primers were available at QIMR for exon 11, 51 and 54 of the ATM gene and the sequences of these are listed in table 4.1.

5μl of each sample was placed in triplicate (for each of the 3 exons) between two 96-well plates. A PCR mastermix was made up with 10X Taq buffer, 25mM MgCl₂, 10mM dNTP and 5units/μl of Taq Gold enzyme. This mastermix was used to make three submaster mixes which included each of the three sets of primers. 20μM of forward and reverse primers were added to the submaster mix making up a final volume of 20μl. This was carefully added to the DNA in the wells of the multiwell plates. PCR was performed under the following conditions; denaturation at 94°C for 10 minutes followed by 25cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 3 minutes. 2μl of the PCR mixture was
examined by agarose gel electrophoresis to determine if the amplification was successful in each of the samples.

**Table 4.1** Forward and reverse primer sequences for screening breast cancer patient’s DNA for specific A-T mutations.

<table>
<thead>
<tr>
<th>EXON</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-11</td>
<td>5'GGCTCAAAAAAAAG'3</td>
<td>3'ACAAGAGATTAAATGACACT'5</td>
</tr>
<tr>
<td>E-51</td>
<td>5'TTTGAGTGATCTTTAGATGTAT'3</td>
<td>3'ACACTCACTCAGTTAACTG'5</td>
</tr>
<tr>
<td>E-54</td>
<td>5'CACTGCAGTGATCTAGACAGT'3</td>
<td>3'CTAGGAAAGACTGAATAC'5</td>
</tr>
</tbody>
</table>

**4.2.3 dHPLC analysis**

dHPLC analysis was performed using a Helix™ system (Varian Inc. Walnut Creek, CA). This system consists of a stationary phase which is 1000 Å alkylated silica encased in a steel tubed column. The DNA samples were carried through the column through the high pressure flow of buffer (Varian bufferPak A) in the mobile phase. An increasing gradient of organic solvent (Varian bufferPak B which contains acetonitrile) caused the small DNA molecules and heteroduplexes to elute before the larger molecules. Star software (Simulation Technology and Applied Research, Inc. Mequon WI) was used for the computational operation and analysis of the process.
**Figure 4.1:** Heteroduplex identification: Star Reviewer software was used to analyse dHPLC chromatograms of PCR products to identify a heteroduplex elution pattern from a series of homoduplexes.

### 4.2.4 DNA sequencing

For DNA samples displaying aberrant dHPLC chromatograms, the PCR was repeated using the same DNA and the same primers and 3:1 big dye terminator mix (Amersham) and the forward and reverse sequences determined using an ABI Prism 377 sequencer.
4.3 RESULTS

4.3.1 PCR Amplification

Figure 4.2 Agarose gel electrophoresis demonstrating the PCR amplification of 28 patient samples for each of the 3 A-T exons (E11, E51 and E54). A 100bp ladder was also placed on the gel.
Figure 4.3 Agarose gel electrophoresis demonstrating the PCR amplification of 29 patients samples for A-T exons (E11, E51 and E54)
4.3.2 dHPLC Analysis

Each of the samples were analysed individually as chromatograms through the star review program. All chromatograms appeared to be normal with the exception of one sample at exon 54. An additional peak was observed in this chromatogram (figure 4.2) to the DNA peaks observed in all of the other chromatograms (Figure 4.3 and Figure 4.4)

**Figure 4.2** Chromatogram showing a heteroduplex peak between 2-3 minutes (at 3 and 8 mvolts) indicating the presence of a mutation at exon 54.
**Figure 4.3** Chromatogram demonstrating only a normal DNA homoduplex peak at 3 minutes for exon 11 in breast cancer sample.

**Figure 4.4** Chromatogram demonstrating a normal homoduplex DNA peak at 3-4 minutes for exon 51 in breast cancer sample.
4.3.3 Sequence Analysis

The mutation which was found initially through dHPLC was confirmed through sequence analysis and it was identified as a heterozygous polymorphism in the intronic sequence that follows exon 54. The polymorphism was IVS54+8G>T and can be observed in the sequence data in figure 4.6.

**Figure 4.6** Sequence data showing the IVS54+8G>T polymorphism found at Exon 54 in a breast cancer sample. It is denoted by the N displayed on the sequence.
4.4 DISCUSSION

There is much literature that documents an increase in breast cancer susceptibility in carriers of the ATM gene. Initial epidemiological studies (reviewed by Easton, 1994) demonstrated that approximately 4% of heterozygote carriers would be attributable to breast cancer cases.

Subsequent studies to determine the ATM mutation types in breast cancer patients were conducted and they confirmed this increased susceptibility. These studies included haplotyping which incorporates the use of genetic markers flanking the ATM gene (Janin et al, 1999) and mutation screening by detection of virtually all mutations - Single-stranded conformation polymorphisms (DOVAM-S) (Sommer et al, 2003; Feng et al, 2003) and denaturing high-performance liquid chromatography (dHPLC) (Berstein et al, 2003; Thorenstenson et al, 2003).

As described previously (section 4.1) initial studies of ATM mutations suggested that approximately 70% of ATM mutations in A-T patients were truncating mutations with 30% being missense mutations. However, this figure was biased as only the protein truncation test was used which possibly also detected null mutations. Thus 70% was most likely to be an over-estimation. A study by Teraoka et al (2001) showed that ATM missense mutations were the most common type in breast cancer cases with only one truncating mutation found amongst 177 breast cancer and 81 control subjects. Therefore missence mutations were the main focus of this study.

Three specific ATM mutations, T7271G, IVS10-6T→G and 7636del9 on exon 51, 11 and 54 respectively were chosen for this study to screen the 57 breast cancer samples through dHPLC analysis. No aberrant chromatograms were detected in all 57 breast cancer patients samples for the T7271G and IVS10-6T→G mutations. Recent published data by Szabo (2004) show mutational screening data of these two specific...
mutations on a large number of subjects (961 families) carried out by various groups. The IVS10-6T→G mutation was detected in only 8/961 families and the T7271G mutation was not detected at all. They concluded that these mutations are not high-risk breast cancer susceptibility alleles and are unlikely to contribute to breast cancer substantially.

In the present study, one chromatogram (1/57) revealed the presence of a mutation on exon 54. Sequence analysis showed that this mutation was not the 7636del9 mutation but a heterozygous mutation in the intronic sequence that follows exon 54. More specifically the mutation was IVS54+8G>T. The A-T mutation database was analysed for this mutation and it was shown to be a mutation previously reported in A-T patients. This mutation was also detected by Teraoka et al (2001) in breast carcinoma patients (1/142) with a first-degree family history or early age at diagnosis. The degree of penetrance of the wide spectrum of A-T mutations in breast cancer susceptibility is still under scrutiny. Interestingly, the patient with the identified mutation was a first-degree relative of another patient in the study which was negative for the mutation. Thus, this IVS54+8G>T A-T mutation did not appear to be a germline mutation in this case.

An observation made by Gatti (1999) was that A-T heterozygotes for missense mutations might predominantly include those who are predisposed to developing sporadic breast cancer. The IVS54+8G>T missense mutation is associated with predisposition of sporadic breast cancer in this patient. The incidence rate of 1/57 patients of this mutation in this study calculates at 1.75% which compares to the incidence rate of 1/142 patients or 0.7% in the Teraoka study. It is possible that due to population heterogeneity that this mutation exists more frequently among
Irish/European breast cancer patients; however a higher number of subjects would be required to screen for this mutation to demonstrate this.

In the recent literature, prevalence of all identified ATM mutations in heterozygote carriers with breast cancer predisposition appear to be low, which illustrates that there is a clear association between ATM and breast cancer predisposition but these mutations are not high-risk breast cancer-susceptibility alleles.
CHAPTER 5

THE RECESSIVE HEREDITARY ATAXIAS: CHARACTERISATION OF A COMPLEX CASE THROUGH CELLULAR AND GENETIC STUDIES

5.1 Introduction
5.2 Materials and methods
5.3 Results
5.4 Discussion
5.1 INTRODUCTION

The hereditary ataxias are a heterogenous group of rare neurodegenerative diseases characterised by progressive cerebellar ataxia associated with a number of different neurological and ophthalmological symptoms. The hereditary ataxias can be subdivided according to their mode of inheritance; autosomal dominant, autosomal recessive and X-linked. Autosomal dominant cerebellar ataxias are classified between the spinocerebellar ataxias (SCA) types 1-16 (reviewed by Di. Donato, 1998). The classification of SCAs are made according to their specific genetic loci (e.g. in identified SCA genes) and clinical features such as onset in adulthood and cerebellar or olivopontocerebellar atrophy.

The autosomal recessive ataxias are characterised according to their genetic background and distinguishing clinical features. This group includes the following ataxias; Friedrich’s ataxia (FRDA), ataxia-telangiectasia (A-T) and ataxia with oculomotor apraxia (AOA) and also much rarer ataxias; ataxia with primary vitamin E deficiency (AVED), abetalipoproteinemia (ABL), spastic ataxia of Charlevoix-Saguenay (ARSASC), infantile onset spinocerebellar ataxia (IOSCA) and Refsum’s disease (RD) (all reviewed by Di Donato, 2001).

Friedrich’s ataxia (FRDA) was first described by Nicholaus Friedreich in 1863. It is the most common form of autosomal recessive ataxia in European countries (DiDonato et al, 2001). The main clinical features are; mean onset at 16 years, progressive ataxia, babinski sign, cardiomyopathy and sensory neuropathy (Durr et al, 1996). FRDA has been associated with the FRDA gene mapped to chromosome 9q13-21 (Campuzano et al, 1996) and encodes a 210-amino acid protein known as frataxin which is necessary for mitochondrial iron-homeostasis.
Ataxia-telangectasia (A-T) is the second most common form of ataxia in childhood after FRDA in European countries. It is characterised by progressive cerebellar ataxia, ocultaneous telangectasia, immunodeficiency, increased serum alphafetoprotein levels, hypersensitivity to ionizing radiation and cancer predisposition. A-T has been associated with the ATM gene which was mapped to chromosome 11q22-q23 (Savitsky et al, 1995) and encodes a 3056-amino acid protein from the phosphatidyl inositol 3-kinase family (Lavin et al, 1995). Ataxia-telangectasia-like disorder (ATLD) has recently been identified and it has many of the clinical features of A-T with the exception of ocular telangectasia. The genetic basis of ATLD has been related to mutation of the hMRE11 gene (Stewart et al, 1999), which is involved in DNA breaks.

The remaining autosomal recessive ataxias are rarer ataxias. Ataxia-oculomotor apraxia (AOA) is one of these syndromes described in patients. Oculor motor apraxia was first described by Cogan et al in 1953, and ataxia-oculomotor apraxia was later described in 1988 by Aicardi et al in a study carried out on 14 families. This primary feature was related to AOA along with other characteristics such as choreoathetosis and sensory and motor neuropathy. Two types of AOA have recently been dissociated; AOAl and AOA2 which are mapped to chromosome 9p13 and 9q34 respectively (See chapter 1 for references). The causative gene for AOAl is known as APTX and it encodes a 342-amino acid and a 168-amino acid protein through alternative splicing of which the protein products are known as long and short aprataxin respectively (though the presence of the short form is disputed). The gene responsible for AOA2 has recently been elucidated. It was called senataxin and was thought to be involved in RNA maturation and termination (Moreira et al, 2004).

Early onset cerebellar ataxia and hypoalbuminemia (EOAH) was initially thought to
be a separate rare autosomal recessive ataxia similar to AOA, however linkage analysis on these patients demonstrating features of AOA1 with hypoalbuminemia was shown to be linked to chromosome 9p13 (Moreira et al, 2001) thus showing that AOA1 and EOAH were the same entity. AOA1 and AOA2 are caused by defects in two different gene products (Moreira et al, 2001, Date et al 2001; Moreira et al, 2004). (See table 5.1) The main distinguishing features between the two syndromes include onset of the disease. Whereas for AOA1 the onset is usually between 2 and 18 years with a mean of 4.7 years, AOA2 has a later age on onset between 10 and 25 years (LeBer, 2004). AOA1 is also associated with hypoalbuminemia after long disease duration and hypercholesterolaemia in contrast to AOA2 which is associated with elevated gamma-globulin, alpha-fetoprotein levels (Le Ber, 2004; Bomont et al, 2000; Nemeth et al, 2000).

A similar autosomal recessive cerebellar ataxia phenotype to AOA1 has recently been identified, which has hypoalbuminemia and hypercholesterolaemia similar to AOA1 but lacks oculomotor apraxia. This ataxia is called sensorimotor cerebellar ataxia with neuropathy (SCAN1) and was linked to a mutation in the TDP 1 gene (Takashima et al, 2002).

Clinical features may be sufficient to distinguish between the various autosomal recessive ataxias. FRDA can be easily distinguished from AOA1 with its distinct clinical features as described previously. However, overlapping clinical features among the recessive ataxias can make the diagnosis of some of these ataxias more difficult. Biological markers are useful for identification and diagnosis. Serum-alpha protein levels are elevated in A-T patients and normal in AOA1 patients, whereas cholesterol and albumin levels may be elevated in AOA1 patients and normal in A-T
patients. However, these features may be variable among a heterogenous population of A-T or AO1 patients.

Further biological tools may also be used in identification and diagnosis of the autosomal recessive ataxias. Elevated radiosensitivity and genomic instability are biological endpoints characterising A-T patients and can be contrasted to normal levels of radiosensitivity in AO1 patients.

In this study, the cells from a patient encoded ATL2ABR were used for the analysis. The patient had previously been clinically characterised. The patient had none of the clinical features of FRDA but had similar and overlapping features to A-T and AO1 and AO2. These are illustrated in table 5.1. The patient ATL2ABR had early age onset, cerebellar ataxia, slow progression, oculomotor apraxia, no detectable sensory and motor neuropathy and no telangetasia. The alpha-fetoprotein levels were tested and were normal (reading 1 which is between the normal range 0-8). Albumin and cholesterol were tested and also appeared normal. However, hypoalbuminaemia and hypercholesterolaemia are not consistent between identified AO1 patients. Furthermore, they are associated with long disease duration. When these clinical tests were carried out, the patient was still very young and was in the early stages of the presenting disease and thus had normal levels of both albumin and cholesterol. From this initial clinical data, it appeared that the patient ATL2ABR was potentially an AO1 patient rather than an AO2 or A-T patient. However, further laboratory based testing on a genetic and cellular level on the patient was required to support this hypothesis.

Further testing included the use of radiation sensitivity as a biological tool for the characterisation of ATL2ABR. Radiation sensitivity was the endpoint measured by the cell survival assay and the G2 cytogenetic assay described in chapter 2 and 3.
Genetic studies included cloning of APTX into an appropriate inducible vector system and subsequent transfection into ATL2ABR lymphoblastoid cell lines which were potentially deficient in APTX and to assess the sensitivity phenotype. Mutational analysis involved the use of dHPLC for screening ATL2ABR for mutations in APTX at various exons as described in chapter 4 for the screening of breast cancer samples for specific A-T mutations.

This data together with functional data carried out by co-workers in the laboratory was poled together and analysed in an attempt to further characterise the cell line and identify the gene responsible for the ATL2ABR patient's autosomal recessive ataxia.
**Table 5.1:** Initial clinical features of the ATL2ABR patient compared to general clinical features of A-T, AOA1 and AOA2 patients.

<table>
<thead>
<tr>
<th>Features</th>
<th>A-T</th>
<th>AOA1</th>
<th>AOA2</th>
<th>ATL2ABR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset</td>
<td>Early (2-15)</td>
<td>Early (2-18)</td>
<td>Late (10-25)</td>
<td>Early - Age 10</td>
</tr>
<tr>
<td>Cerebellar Ataxia (CA)</td>
<td>CA</td>
<td>CA</td>
<td>CA</td>
<td>CA</td>
</tr>
<tr>
<td>Progression</td>
<td>Fast</td>
<td>Slow</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td>Oculomotor Apraxia (OA)</td>
<td>OA</td>
<td>OA</td>
<td>OA</td>
<td>OA</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>Sensory + Motor</td>
<td>Sensory + Motor</td>
<td>Sensory + Motor</td>
<td>Sensory + Motor</td>
</tr>
<tr>
<td>Oculaneous Telangectasia (OT)</td>
<td>OT</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Alpha-fetoprotein</td>
<td>Elevated</td>
<td>Normal</td>
<td>Elevated</td>
<td>Normal</td>
</tr>
<tr>
<td>Albumin</td>
<td>Normal</td>
<td>Decreased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Normal</td>
<td>Increased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>
5.2 MATERIALS AND METHODS

5.2.1 Patient cells

10-20 ml of whole blood was extracted from the patient (who was from Sydney) and sent to the Queensland Institute of Medical Research (QIMR), Brisbane. Lymphoblastoid cell lines (LCLs) were made by isolating the lymphocytes on a ficoll-paque (Gibco™ Invitrogen Corporation, Carlsbad, CA, USA) gradient and transforming them with EBV virus as described in chapter 3. The cells were coded ATL2ABR and they were maintained in RPMI 1640 medium (Gibco) supplemented with 10% v/v foetal calf serum (20IU/20μg per ml) and 10mM L-Glutamine (Both Gibco).

5.2.2 Radiation Sensitivity Assays

Radiation sensitivity was measured by cell survival and the G2 cytogenetic assay. An A-T LCL (AT1ABR) and a control LCL (C3ABR) were also used to compare against ATL2ABR LCL radiosensitivity results. For the cell survival assay; ATL2ABR cells were counted and plated at 2 x 10^5 per ml per flask set up in triplicate per dose point. The cells were incubated overnight at 37°C in a 5%CO₂ environment. The cells were irradiated with 0.5Gy and 1Gy of gamma radiation, and the control cells were sham-irradiated. The cells were placed back into the incubator for 48hours. 0.5ml of well-mixed cells were added to 0.1ml of trypan blue dye (0.4%) (Gibco). The viable cells (the unstained cells) were counted on the centre grid of a haemocytometer (on both ends) and a mean number calculated. The percentage survival fraction was calculated for each dose point taking the survival fraction of the control (0Gy) into consideration as the control was equal to 100%. The formula was as follows:
The number of cells counted/ml * Survival fraction of the control * 100
The number of cell initially plated/ml 1

The G2 chromosomal radiosensitivity assay was also used to characterise these LCLs. The cells were plated approximately one in two to ensure that they were in the log phase of growth. They were incubated at 37°C at 5% CO₂ for 24 hours. The cells were then irradiated with 0.5 Gy of gamma radiation. The controls were sham-irradiated. The cells were placed back into the incubator for 30 mins post irradiation. Colcemid (Gibco) at 1 mg/ml was then added to the cells for 60 minutes to halt the cells in metaphase. Chromosome harvesting was then carried out as described in Chapter 2 and 3. Slides were prepared and analysed as previously described.

5.2.3. Molecular cloning and expression of aprataxin in LCLs

A. PCR amplification of APTX DNA:

Two forward primers and one reverse primer were designed to amplify both the long and short form of aprataxin (See table 5.2 for primer sequences). A Not1 restriction site was incorporated into the design of both forward primers and an Xho1 restriction site was incorporated into the design of the reverse primer. Plasmid DNA containing both the Long and short form of the APTX cDNA was used as the DNA template in the PCR reaction. The PCR reaction mixture was as follows; 100 ng/μl concentration of template DNA, 100 ng/μl of forward and reverse primer (Sigma-Genosys, TX, USA), 300 μM each dNTPs, 2.5 U/μl Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the 10X cloned Pfu DNA polymerase reaction buffer supplied, and finally distilled water was added to the reaction to complete the total reaction.
volume (100μl). The primer annealing temperature $T_a$ was calculated at
approximately 64°C for this reaction. Thus, the PCR reaction was performed under
the following conditions; denaturation at 96°C for 1 minute followed by 25 cycles of
denaturation at 96°C for 1 minute, annealing at 64°C for 1 minute and extension at
72°C for 1 minute/kb. 2μl of the PCR reaction was examined by agarose gel
electrophoresis to determine whether the correct sized products were obtained.

Agarose gel electrophoresis involved making a 1% Agarose gel (Progen Industries
Ltd, Darra Queensland, AUS) in TAE buffer (See appendices). The samples were run
in the gel in a DNA gel rig (Jordan Scientific, Bloomington, IN, USA) at 80Volts for
20-30 minutes. Two DNA ladders were used on DNA gels throughout this study for
estimation of DNA bands; a 100 base pair (bp) ladder (New England Biolabs® Inc,
Beverly, MA, USA) which included DNA fragments that ranged from 100 – 1,217bp
and a lambda DNA – Hind III digest ladder (New England Biolabs® Inc) which had a
wider range of DNA fragments from 2,027-23,130bp.

A larger volume of the PCR mixture (20μl) was placed on another 1% agarose gel
with wider combs to get distinct clear bands. The appropriate bands (Approximately
1kb for long $APTX$ and 0.5kb for short $APTX$) were excised from the gel and placed in
ependorf tubes. The weight of each gel slice was recorded. The PCR product was
purified from the agarose gel using a perfectprep® Gel cleanup kit (Eppendorf GmbH,
Hamburg, Germany). This involved adding 3 volumes of the kits binding buffer for
every one volume of gel slice where 1mg equals 1μl of volume. The gel pieces were
completely dissolved by incubation on a heating block at 50°C for 5-10 minutes. One
volume of isopropanol (BDH Industries, Quebec, Canada) equal to the original slice
volume was then added to the samples to precipitate the DNA. The sample was then
placed into a column placed in a 2ml collection tube (provided by the kit) and spun in
a microcentrifuge at 6,000 – 10,000 x g for 1 minute so that the DNA was collected in
the column. The column was washed with wash buffer that was supplied by the kit
(diluted with ethanol (BDH)). The DNA was then eluted into a new eppendorf
collection tube with 30µl of the supplied elution buffer. The DNA was then ready for
subsequent cloning.

B. Cloning of short APTX (S.APTX) and long (L.APTX) into vector systems

Cloning of both S. APTX and L.APTX was first carried out using pGEM®-T Easy
Vector which was supplied as a kit (Promega Corporation, Madison, USA). pGEM-T
Easy is a convenient system for cloning PCR products. It is a 3015bp vector with T7
and SP6 RNA polymerase promoters flanking a multiple cloning region (with
multiple restriction sites) within the α-peptide coding region of the enzyme β-
galactosidase. Insertional inactivation of the α-peptide allows the identification of
recombinant clones through colour selection. See Figure 5.1 for a diagrammatic
representation of this vector system. The purified PCR DNA from above was
quantified on a spectrophotometer at 260nm wavelength. The amount of DNA for a
subsequent ligation reaction was calculated (See appendix) for a 3:1 insert:vector
molar ratio. These were added to the remaining ligation reaction of; 2X rapid
Ligation buffer, T4 DNA Ligase (3 Weiss units/µl) and deionised water to make a
final volume of 10µl. The reactions were incubated overnight at 16°C.

1/2µl of the ligation reaction was added aseptically to a 100µl aliquot of pre-prepared
DH5α competent bacterial cells (Invitrogen, Carlsbad, CA, USA) for transformation
(see appendices). The DNA and cells were mixed gently and placed on ice for 20
minutes. The mixture was heat-shocked at 42°C for 45 seconds and returned to ice for
2 minutes. 200µl of Luria Broth (LB) media (Gibco) was added to the mixture before
a further incubation at 37°C with shaking for 45 minutes. The samples were then spun gently for 20 seconds and the supernatant pipetted off. The cell pellet was resuspended in 100μl of LB media and plated onto pre-warmed LB plates (Gibco) with ampicillin (0.1mg/ml) (Gibco) supplemented with 100mM of Isopropyl-beta-D-thiogalactoside (IPTG) (Gibco) and 50mg/ml of X-galactose (X-Gal) (Gibco) (see appendices). After incubation of the plates at 37°C overnight, resulting colonies were examined on each plate. Colony selection in this system enabled the visual detection of bacteria containing the plasmid with insert (white colonies) which occurs due to the interruption of the β-galactosidase gene in the vector in contrast to bacteria containing the empty plasmid (blue colonies). These colonies were incubated at 37°C with shaking overnight to increase the numbers of bacterial cells and therefore the amount of plasmid DNA. The DNA was then extracted from the bacterial cells using a Qiaprep® spin miniprep kit (Qiagen GmbH, Hilden, Germany). This involved lysis of cells and extraction of plasmid DNA though supplied buffers P1, P2 and N3. The samples were centrifuged for 10 minutes at 13,000rpm to pellet the cells leaving the DNA in the supernatant. The supernatant was added to a column in a 2 ml collection tube and centrifuged for 1 minute at 13,000rpm to allow the DNA to bind to the column. The column was washed with the supplied wash buffer (diluted with ethanol (BDH)) and the DNA was eluted with 30μl of the kits elution buffer. An aliquot of the purified plasmid DNA was examined by agarose gel electrophoresis to check the plasmid preparations for contamination etc.
Table 5.2 Sequences of forward and reverse primers for cloning \textit{S.APTX} and \textit{L.APTX} into pGem-T Easy and HA-tagged pMep4 vector systems

<table>
<thead>
<tr>
<th>Cloning Vector</th>
<th>Primer</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGem-T Easy</td>
<td>Long\textit{APTX} – Forward Primer</td>
<td>5‘GCGGCCGCGATGATGCGGGTGTGCTGG’3</td>
</tr>
<tr>
<td></td>
<td>Short\textit{APTX} – Forward Primer</td>
<td>5‘GCGGCCGCGCTATGCAGGACCCAAAAATGC’3</td>
</tr>
<tr>
<td></td>
<td>\textit{APTX} – Reverse Primer</td>
<td>3‘CTCGAGTCACTGTGTCCAGTGCTTCC’5</td>
</tr>
<tr>
<td>HA-pMep4</td>
<td>Short\textit{APTX} – Forward Primer</td>
<td>5‘ATACTCGAGAATGCAGGACCCAAAAATGC’3</td>
</tr>
<tr>
<td></td>
<td>Short\textit{APTX} – Reverse Primer</td>
<td>3‘AGCGGATCTCTCAGTGCCAGTGCTTCC’5</td>
</tr>
</tbody>
</table>

Figure 5.1 pGemT-Easy and pMep4 vector maps

C. Restriction Enzyme digestions of plasmid DNA to confirm correct cloning of \textit{APTX} insert DNA.

In the initial amplification of the \textit{L.APTX} and \textit{S.APTX} cDNAs the primer design included the restriction sites Not1 and Xho1. These enzymes were used in the restriction enzyme digestion of purified plasmids to excise the cloned band from the
plasmid and interpret the band based on its molecular size in base pairs (bp). The restriction enzyme digestion reaction was as follows; 2U of NotI and 2U of XhoI (New England Biolabs), 100X BSA and 10X buffer 3 (both supplied with enzymes) and deionised water to make up the final reaction volume. The digestion reaction was incubated at 37°C for 2 hours. Half of the digestion reaction was examined by agarose gel electrophoresis to determine if the correct APTX product was obtained.

D. Sequencing of correct APTX products

Each of the samples which showed the correct sized products were quantified by spectrophotometric analysis at a wavelength of 260nm. Sequencing reactions were set up and consisted of; 200-500ng concentration of double-stranded DNA, 3.2pMol concentration of either T7 or SP6 primers. (The forward primer T7 (Sigma-Genosys) was used for forward sequencing reactions and the primer SP6 (Pro-Oligo, La Jolla, Ca, USA) was used for reverse sequencing reactions), Version 3:1 big dye terminator matrix (ABI, Amersham Uppsala, Sweden) and deionised water to a final volume of 10μl. The sequencing PCR reaction was performed under the following conditions; denaturation at 96°C for 1 minute followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes. The amplified DNA was precipitated from the PCR mixture with 70% isopropanol (BDH) and centrifugation steps. The forward and reverse PCR sequences were determined using an ABI Prism 377 sequencer (Amersham). The resulting DNA sequences were analysed electronically through searches using the BLAST database.
E. Sub-cloning of *APTX* from pGem-T easy vector into pMep4 (inducible) vector system for subsequent expression in lymphoblastoid cell lines

Once the correct sequences of aprataxin in pGem-T were obtained, the *APTX* inserts were cut out of this vector and cloned into the inducible vector system known as pMep4.

pMep4 (Invitrogen) is a 10.4Kb vector with an origin of replication, a large multiple cloning site (with multiple restriction sites) and ampicillin resistance for selection in bacterial cells. It is suitable for application in mammalian lymphoblastoid cell lines as it contains an EBV protein coding region (EBNA-1), hygromycin gene for efficient selection in eukaryotic cells, and a metallothionein promoter (P hMTIla) to induce the expression of cloned cDNA. See Figure 5.2 for a diagrammatic representation of the pMep4 vector system.

The purified pGem-T Easy plasmids (sequence verified) were digested as described above with NotI and XhoI restriction enzymes. The entire digestion reaction (20μl) was placed on a 1% agarose gel and the correct band (*APTX* inserts) was excised from the gel and the DNA purified with a perfectprep® Gel clean-up Kit (Eppendorf) as described previously. Meanwhile, the pMep4 vector was also digested as above with NotI and XhoI to create sticky ends in the vector for cloning in the *APTX* inserts.

The plasmid digest was also gel purified as described above. A ligation reaction was set up to ligate the purified *APTX* inserts into the purified pMep4 vector. The ligation reaction was the same as that explained above (section B) and was incubated at 4°C overnight. 2μl of the ligation reaction was transformed in DH5α competent cells as described previously, however this vector system did not have colour selection to identify the recombinant clones. 2 colonies were aseptically picked from each plate and grown in 5 mls of LB media at 37°C overnight with shaking. Mini prep DNA
extractions were carried out on the bacterial cultures using a Qiaprep® spin miniprep kit (Qiagen) also described in more detail above. The purified plasmids were then digested with the same restriction digestion reaction above and examined by agarose gel electrophoresis to determine if the correct sized band (s) or APTX insert had been cloned into pMep4 vector. Complete confirmation of correct cloned inserts in pMep4 was obtained after sequence analysis. Subsequently the sequence verified DNA was grown in bacterial cells in larger (250ml) culture volumes (1ml was retained to make bacterial culture glycerol stocks (see appendices)) and then extracted using a perfectprep® plasmid midi kit (Eppendorf). This protocol was the same in principle as the qiaprep® spin miniprep kit except it was designed to accommodate larger volumes (50-250ml). It involved lysing the cells with buffer1 and 2 and neutralisation with buffer 3. The solutions were centrifuged at 15,500g for 15 minutes in a high-speed centrifuge (Beckman Coulter™ Avanti (Beckman Instruments Inc ©, Fullerton, CA, USA)) and the supernatant containing the DNA was carefully poured off using a sieve to remove any attached cellular debris. Cold DNA binding matrix (which was mixed well by vortexing prior to use) was added to the supernatant to specifically bind the DNA. The solution was then added to a spin-column to bind the DNA-matrix to the column. The matrix spin-column assembly was centrifugated at 2000g for 5 minutes in a Beckman J-6M/E centrifuge (Beckman Instruments Inc ©). The column was then washed with 10ml of diluted purification solution (diluted in the specified amount of ethanol). The column was dried briefly through centrifugation of the column for 5 minutes at 2000g. Elution of DNA from the column was carried out by placing the column into a new centrifuge tube and adding 3mls of the supplied elution buffer to the column and further centrifugation. This protocol included final DNA purification steps after these initial elution steps, however spectrophotometric analysis
demonstrated high purity of DNA (approximately 1.8-1.9) and consequently these steps were omitted.

**F. Cloning of APTX inserts into pre-HA tagged pMep4 Vector.**

No antibodies for aprataxin were commercially available at the time, therefore it was decided that using a Haemaggulutinin (HA) protein-tagged pMep4 vector system (provided by Dr. Shaun Scott (QIMR)) would enable further identification of the aprataxin in transfected cells. A new set of primers were designed which incorporated the XhoI and BamHI restriction sites. See table 5.2 for primer sequences. The PCR reaction was as follows; 100ng/μl of sequence verified pGem-T easy with APTX inserts was the DNA template, 300μM dNTPs, 100ng/μl of both forward and reverse primers (Sigma-Genosys), 2.5U/μl Pfu turbo™ DNA polymerase and 10X buffer supplied (Stratagene) and deionised water to a total reaction volume of 100μl. The PCR was performed as described previously for initial cloning of APTX into pGem-T Easy. The DNA was precipitated out of the PCR reaction by ethanol concentrations. 2-3 volumes of ice cold 100% ethanol (BDH) and 1/10 of the total volume of sodium acetate (NaAc) (BDH) pH 5.2 was added to the PCR mixture and mixed well by vortexing. The samples were incubated at 4°C for 30 minutes and then centrifuged at 4°C, 13,000rpm for 20 minutes. The supernatant was carefully taken off and the pellet was washed with 1ml of 70% ethanol and centrifuged as above. The supernatant was carefully discarded and the DNA pellet was allowed to air dry for 15-20 minutes. The pellet was then resuspended in 30μl of deionised water. The Cloning methods which followed were the same as those described for pGem-T Easy and pMep4; Restriction Digestion was carried out on the precipitated DNA with XhoI and BamH1 restriction enzymes (New England Biolabs® Inc) in parallel with the HA-
tagged pMep4 to create sticky ends. Subsequent ligation of the \textit{APTX} cDNA into the HA-tagged pMep4 vector was carried out and transformation into DH5\textalpha{} competent cells by heat shock. Clones containing the insert were identified by restriction digest and the integrity of the cDNA was additionally verified by sequencing. Subsequently, large bacterial culture volumes (250-500 ml) were grown at 37°C for 24 hours. The DNA was extracted with the perfectprep\textsuperscript{®} plasmid midi kit (Eppendorf) and concentration and purity was determined spectrophotometrically for transfection into ATL2ABR cells.

\textbf{G. Transfection of pMep4 and HA-tagged pMep4 plasmids with APTX inserts into ATL2ABR lymphoblastoid cell lines.}

Transfection of ATL2ABR cells with pMep4 plasmids containing \textit{SAPTX} and \textit{LAPTX} was performed through electroporation. Approximately \(10^7\) cells were washed once with RPMI-1640 media (Gibco\textsuperscript{™}) without any supplements. The pelleted cells were then resuspended in 500\,\mu{l} of the same media. Meanwhile, 20\,\mu{g} of plasmid DNA was placed in a 0.4\,cm electrode distance gene pulser\textsuperscript{®} cuvette (Bio-Rad laboratories, Hercules, CA, USA). The resuspended cells were also added to the cuvette, mixed and incubated at room temperature for 10 minutes to allow the cells to adjust to the environment. The cuvette was then placed into an ECM\textsuperscript{®}830 Electroporator (BTX Genetronics Inc, San Diego, CA, USA) and the cells were exposed to a high electric field of pulses for a short duration (200\,V for 10\,mSec) to create temporary pores in the cell membranes that enables the plasmid DNA to enter the cell. The cuvette was immediately placed on ice and incubated for 10 minutes to allow recovery of the membranes of the cells. The cells were then resuspended in 25\,ml of RPMI-1640 supplemented with 10\% v/v foetal calf serum, (20\,IU/20\,\mu{g} per
ml) penicillin/streptomycin and 10mM L-Glutamine (all Gibco™) and 1ml of the suspension placed into each well of a 24-well plate (Falcon, St. Louis, MO, USA). The plate was incubated at 37°C with 5% CO₂ for 24 hours. Hygromycin (Gibco™) was then placed onto the cells in concentrations varying from 50 - 200μg/ml to select for the cells that only contain the plasmid. Hygromycin-containing media was changed twice a week for approximately 3 weeks to ensure complete selection of cells.

H. Induction of Aprataxin protein in transfected ATL2ABR cells

Confluent transfected ATL2ABR cells were subcultured by diluting them 1:2 in cell culture media (RPMI-1640 (Gibco) supplemented with 10% foetal calf serum (Gibco) and 10mM L-glutamine (Gibco)) 24 hours before use to ensure that they were in the log phase of cell growth. The cells were incubated at 37°C with 5% CO₂. They were then further subcultured 1:1 in media and then 10mls dispensed into each flask set up per dose point. They were incubated at 37°C with 5% CO₂ for a further 8-10 hours. Concentrations from 0-10μM of Cadmium Chloride (CdCl₂) (Sigma-Aldrich, St-Louis, MO, USA) were added to the cells from a 5mM stock solution, to induce APTX in the HA-pMep4 vector contained within the cells. The cells were incubated at 37°C for 16 – 24 hours to ensure induction.

I. Functional Studies – Protein analysis

Protein sample preparation:

The cells were centrifuged at 1200rpm for 10 minutes and washed once with phosphate buffered saline (PBS) (See appendices) and transferred to eppendorf tubes
(Eppendorf). 100-200μl of protein lysis buffer (Tris-buffer 1 with NP-40 detergent) (See appendices) with dithiothreitol (DTT), phenylmethylsulfonylfluride (PMSF), sodium fluoride (NaF) and sodium vanidate (NaVa) added just before use was added to the cell pellet depending on size. The cells were incubated on ice for 45 minutes. The lysed cells were subjected to further lysis through sonication (Model 250/450 Sonifier®, Branson Ultrasonics, CT, USA) at 4°C. Two short pulses were applied to each sample and they were then centrifuged at 13,000rpm for 20 minutes. The supernatant was carefully transferred to new eppendorfs. Protein estimation was carried out on the supernatant using a multowell-plate (Falcon) the Bio-Rad Dc protein assay kit (Bio-Rad) according to the manufacturer’s instructions in a multiwell-plate format (Falcon, St. Louis, MO, USA). The protein concentration was obtained by reading the samples at 705nm on a micro-plate reader using the soft-max pro plate reader computer program. The samples were prepared at a concentration of 50μg/μl and protein loading dye (see appendices) which contains mercaptoethanol as the active ingredient was added to a volume suitable for the wells in the PAGE gel. The samples were then placed in a heat block at 100°C for 10 minutes to denature the proteins.

**Polyacrylamide Gel electrophoresis (PAGE) and Western Blotting:**

Depending on the size of the proteins to be detected, 10% or 12% polyacrylamide gels were used to separate the proteins in the above samples (see appendices). A 12% gel was made to separate proteins of lower molecular weight such as the SAPTX (approximately 25kDa protein). The separating gel was made up with 30% acrylamide (Bio-Rad laboratories), 1.5M Tris solution pH 8.8 (BDH, Poole, UK), 10% Sodium Dodecyl Sulphate (SDS) (Sigma-Aldrich), 10% APS, N’-
Tetramethylethylenediamine (TEMED) (Sigma-Aldrich) and water to a final desired volume for the electrophoresis apparatus. A 4% stacking gel was then made up with the same constituents as above except the 1.5M Tris solution (BDH) was replaced with a 1M Tris solution at pH 6.8. (See appendices). The samples were loaded carefully onto the gel leaving the end lanes free for loading protein markers. The main protein marker used was Benchmark™ pre-stained ladder (Invitrogen) because of the range of proteins varying from 10-200kDa. The fermentas pre-stained protein ladder (MBI Fermentas Inc©, Newington, NH, USA) has a range of proteins from 10-180kDa and was also used occasionally. The samples were run through the gel with PAGE running buffer (See appendices) at a constant electrical current of 20mA per gel.

The separated immobilised proteins on the PAGE gel were then transferred / blotted onto a hybond C nitrocellulose membrane (Amersham-Pharmacia Biotech). The gel was placed on top of the membrane and two pieces of blotting paper were sandwiched each side. The proteins were transferred from the gel onto the membrane through an electrical current of 100 volts for 1 hour in a tank transfer apparatus (Bio-Rad laboratories) with high molecular weight transfer buffer (see appendices).

**Identification of proteins by immunoblotting using specific antibodies:**

Non-specific protein binding on the membrane was blocked by incubation in 5% non-fat milk powder in tris buffered saline with 1% tween 20 solution (TBS-T) (see appendices) for 1 hour with shaking at room temperature. Meanwhile, the primary antibody (IgG) was diluted in a 5% milk protein solution in TBS-T. Two main antibodies were used for the identification of $APTX$ in both the pMep4 vector and HA-pMep4 vector. A specific $APTX$ IgG was made by an associate in the QIMR
laboratory. This was accomplished through the gluthathione S-transferase (GST) gene fusion system (Amersham-Pharmacia Biotech, Denver, CO, USA) which included the cloning of APTX into the GST gene fusion vector (pGex-5X-1) and subsequent regular injections of the tagged recombinant proteins into sheep, to induce high levels of APTX IgG production. The APTX IgG’s were then extracted at regular intervals from the blood of the sheep. The secondary antibody used with this primary APTX IgG was goat-anti sheep IgG (Roche Diagnostics GmbH, Mannheim, Germany). The second antibody that was used for this study was commercially produced; anti-HA high affinity rat monoclonal IgG (Clone 3F10) (Roche Diagnostics) and it was specific for the HA tagged vector system (HA-pMep4). The secondary antibody used with this HA antibody was a peroxidase-conjugated affinity-pure goat anti-rat IgG (Jackson Immuno research labs Inc, Baltimore, USA). The primary antibody was made up in 5% milk protein powder. APTX-IgG was diluted 1:40,000 and HA-IgG was diluted 1:10,000. The diluted antibodies were added to the membranes and incubated overnight at 4°C with shaking. After washing three times with TBS-T buffer for 10 minutes each to remove unbound antibody, the secondary antibody was diluted in 5% milk protein solution (1:8,000 for anti-sheep IgG and 1:10,000 for anti-rat IgG) and was incubated on the membranes for 1 hour at 37°C with shaking. The membrane was washed as above. Protein bands were visualised using an ECL chemiluminescence detection kit (Amersham) according to the manufacturers’ instructions, and the blot which was exposed to X-ray film (Bio-rad) was then developed by an X-Omat machine or manually.
5.2.4 dHPLC analysis

dHPLC analysis was carried out on ATL2ABR cells by Dr. Geoff Birrell (QIMR). DNA was extracted from the ATL2ABR cells and used as a template in the PCR amplification method. Various primers were used to span the exons of both LAPTX and S.APTX for associated mutations analysed by dHPLC. The exons of APTX screened and associated primers used for this analysis can be observed in table 5.3. PCR and dHPLC were performed as described in chapter 4, section 4.2. dHPLC analysis was performed using a Helix™ system (Varian Inc. Walnut Creek, CA). Star software (Simulation Technology and Applied Research, Inc. Mequon WI) was used for the computational operation and analysis of the process.

5.2.5 Additional protein analysis

Various protein expression and analysis studies were carried out on ATL2ABR by Dr. Nuri Gueven (QIMR) to identify the underlying disease in ATL2ABR. Levels of ATM protein and other proteins such as JNK, ser15, ser20 were assessed. ATM and chk2 kinase activity as well as p53 stabilisation in ATL2ABR were analysed.

Table 5.3  Exons of APTX screened for mutations in ATL2ABR cells through dHPLC analysis and the primer sequences used.

<table>
<thead>
<tr>
<th>APTX Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 2/3</td>
<td>5’aagataggagcaagtgcctgg’3</td>
<td>5’atcatctaccaatcacactaccc’3</td>
</tr>
<tr>
<td>Exon 4</td>
<td>5’cacacagtttaactgttgcc’3</td>
<td>5’ttgttctacagaacagccc’3</td>
</tr>
<tr>
<td>Exon 5</td>
<td>5’cttagtgatataacaccaggc’3</td>
<td>5’ctgatctatctgccccacctgg’3</td>
</tr>
<tr>
<td>Exon 7</td>
<td>5’ggetgtagtagtactgtccg’3</td>
<td>5’ttatccaaatttatttcaggg’3</td>
</tr>
</tbody>
</table>
5.3 RESULTS

5.3.1 Radiosensitivity of ATL2ABR cells determined by cell survival and the G2 chromosomal radiosensitivity assay

Since there was an overlap in the symptoms between ATL2ABR patient and A-T patients, sensitivity to ionising radiation was determined. Previous data have shown that A-T cells are hypersensitive to this agent.

The data in figure 6.1 demonstrate that ATL2ABR cells are intermediate in their survival to ionizing radiation. Intermediate radiosensitivity of ATL2ABR to the C2ABR control cells was observed. Although a marked difference in radiosensitivity was observed in ATL2ABR from the control, it was not to the same extent as the both of the A-T patients which show hypersensitivity. This is illustrated in figure 5.1. The error bars at each point on the survival chart indicate the standard error (see appendices for details).

When this was compared with the G2 chromosomal radiosensitivity assay, the results correlated well demonstrating this intermediate radiosensitivity. The G2 scores of the control C2ABR and C3ABR were 112 and 106 aberrations per 100 metaphases respectively. The G2 scores of the A-T cells AT1ABR and AT25ABR were 371 and 356 aberrations per 100 metaphases. These scores were compared to the G2 score of ATL2ABR cells which was 174 aberrations per 100 metaphases, further demonstrating this intermediate radiosensitivity. These radiation induced aberration yields (G2 scores) are illustrated in table 5.4. and 5.5.
Figure 5.2 % Cell Survival of ATL2ABR cells in comparison to the % cell survival of A-T cell lines (AT25ABR and AT1ABR) and a control (C2ABR) cell line in response to ionising radiation.
Figure 5.3 G2 chromosomal radiosensitivity scores (expressed as % aberrations per 100 metaphases counted) of ATL2ABR cells in comparison to the G2 scores calculated in A-T (AT1ABR and AT25ABR) and control (C2ABR and C3ABR) cells.

Table 5.4 Radiation induced aberration yields (expressed as mean ± standard deviation of the triplicate mean values) in the ATL2ABR patient in comparison to A-T (AT1ABR and AT25ABR) and control (C2ABR and C3ABR) cell lines.

<table>
<thead>
<tr>
<th>LCL</th>
<th>Radiation-induced aberration yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2ABR</td>
<td>112.3 ± 4.2</td>
</tr>
<tr>
<td>C3ABR</td>
<td>106 ± 6.6</td>
</tr>
<tr>
<td>ATL2ABR</td>
<td>173.7 ± 16.4</td>
</tr>
<tr>
<td>AT1ABR</td>
<td>371.3 ± 27.2</td>
</tr>
<tr>
<td>AT25ABR</td>
<td>356.3 ± 31.6</td>
</tr>
</tbody>
</table>
5.3.2 Cloning of *APTX*

**Figure 5.4** Agarose gel electrophoresis of restriction digest of *LAPTX* and *SAPTX* cloned into pGemT-Easy vector system.

![Agarose gel electrophoresis of restriction digest of LAPTX and SAPTX cloned into pGemT-Easy vector system.](image)

- 3400bp
- 900bp
- 500bp

- pGemT-Easy Vector
- *LAPTX* insert
- *SAPTX* insert

**Figure 5.5** Agarose gel electrophoresis of restriction digest of *LAPTX* and *SAPTX* cloned into pMep4 vector system.

![Agarose gel electrophoresis of restriction digest of LAPTX and SAPTX cloned into pMep4 vector system.](image)

- 10.4Kb
- 0.9Kb
- 0.5Kb

- pMep4 Vector
- *LAPTX* insert
- *SAPTX* insert
5.3.3 Induction of \textit{SAPTX} in pMep4 in ATL2ABR cells

Expression of \textit{SAPTX} in pMep4 was induced in the transfected ATL2ABR cells with 1-5\textmu M of Cadmium chloride after 16 hours. The blot was probed with anti-HA antibody for detecting the induced short form of \textit{APTX} in pMep4 in ATL2ABR cells. The induction appeared concentration-dependent (See figure 5.6). No band was observed in the control (0) concentration in contrast to the appearance of a band at the first concentration of 1\textmu M. This band appeared to be stronger with increasing concentration from showing a band two-fold bigger with double the concentrations between 1-2.5\textmu M and 2.5-5\textmu M. Therefore 5\textmu M of Cadmium Chloride was used to induce the transfected cells for the subsequent time-point induction experiment. In this experiment, it was demonstrated in ATL2ABR cells that aprataxin was first detectable at 2 hours after addition of 5\textmu M Cadmium chloride and expression increased further between 2-4 hours. Induction increased slightly at each time-point between 4-12 hours (the latter showing the highest induction). This is illustrated in figure 5.7.

\textbf{Figure 5.6} Concentration-dependent induction of \textit{SAPTX} in pMep4 in ATL2ABR cells using cadmium chloride at concentrations up to 5\textmu M and western blot analysis using HA-tag specific antibody.
**Figure 5.7** Time-dependent induction of S.APTX in pMep4 vector in ATL2ABR cells using 5μM Cadmium chloride at various time-points between 0-12 hours and western blot analysis using HA-tag specific antibody.

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>25kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Short aprataxin (all bands)

---

5.3.4 Cell Survival of ATL2ABR cells transfected with short *APTX*.

The radiosensitivity of ATL2ABR cells transfected with induced short *APTX* was compared to the radiosensitivity of A-T (AT25ABR) and control (C2ABR) cells with the same doses of ionising radiation as carried out previously (see figure 5.8). The A-T cells (AT25ABR) displayed a two-fold increased radiosensitivity compared to the control cells (C2ABR). These radiosensitivity values were similar to those observed in figure 5.2. Thus, no significant difference in radiosensitivity between the transfected and un-transfected ATL2ABR cells could be detected.
Figure 5.8. % cell survival of ATL2ABR cells (with cadmium chloride induced S.\textit{APTX} in pMep4) in comparison to A-T cells (AT25ABR) and control cells (C2ABR).

5.3.4 Denaturing high-performance liquid chromatography (dHPLC) analysis
dHPLC was carried out to screen the DNA extracted from ATL2ABR cells for mutations in \textit{APTX}. No aberrant heteroduplex peaks were found in any the chromatograms analysed from dHPLC analysis. Only homoduplex peaks were observed in each sample (as described in chapter 4). Thus, no mutations were detected in all of the screened exons of aprataxin in ATL2ABR cells in this analysis.
5.3.5 Additional Results on the ATL2ABR patient

Additional data on the ATL2ABR patient were obtained from the principal investigator (Dr. Nuri Gueven) of this study through various protein assays and tests that were carried out on ATL2ABR. These are illustrated in table 5.5. In addition, when compared to control cells, a ten-fold increased sensitivity to the crosslinking agent mitomycin C was detected in ATL2ABR cells.

**Table 5.5:** Protein/Enzyme data of ATL2ABR patient

<table>
<thead>
<tr>
<th>Protein/Enzyme Analysis</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM Protein levels</td>
<td>Normal</td>
</tr>
<tr>
<td>ATM Kinase Activity</td>
<td>Normal</td>
</tr>
<tr>
<td>Chk2 Kinase Activity</td>
<td>Normal</td>
</tr>
<tr>
<td>JNK Protein levels</td>
<td>Low</td>
</tr>
<tr>
<td>P53 stabilisation</td>
<td>Delayed</td>
</tr>
<tr>
<td>Ser15 phosphorylation</td>
<td>Low</td>
</tr>
<tr>
<td>Ser20 phosphorylation</td>
<td>Low</td>
</tr>
<tr>
<td>Nbs1 protein levels</td>
<td>Normal</td>
</tr>
<tr>
<td>Mre11 protein levels</td>
<td>Normal</td>
</tr>
<tr>
<td>Rad50 protein levels</td>
<td>Normal</td>
</tr>
<tr>
<td>MRN protein complex levels</td>
<td>Normal</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION

The hereditary ataxias represent a heterogeneous group of rare neurodegenerative disorders. The main ataxias discussed in this study are those of autosomal recessive inheritance and they are classified according to their distinct clinical features and genetic background.

This ongoing case study presents a patient who first showed clinical symptoms of a recessive ataxia at the early age of 10 years. Clinical diagnosis and tests were initially carried out on this patient to determine the condition. Although there were some overlapping clinical features of the patient with Ataxia-telangiectasia such as early onset, cerebral atrophy and oculomotor apraxia, other features indicated that perhaps another recessive ataxia was responsible. The clinical features of this patient appeared to be closer to the description of the AOA1 phenotype taking into consideration that alpha-fetoprotein, albumin and cholesterol are variables in the diagnosis of the disease. Furthermore, the disease was at a very early stage in the patient to test these biological parameters correctly.

The main objective of this study was to investigate if the patient was a true AOA1 case. Cellular and molecular analysis was carried out on the patients cells (encoded ATL2ABR) for this investigation. APTX gene was transfected into the ATL2ABR cells and the sensitivity phenotype recorded. Mutational analysis was also carried out using dHPLC analysis to screen the patients DNA for mutations in the APTX gene.

Cellular radiation sensitivity studies were carried out on ATL2ABR cells through the cell survival assay and G2 chromosomal radiosensitivity assay. Moderately increased radiosensitivity was observed in both of the assays in ATL2ABR in comparison to the control cells, and this contrasted to the radiosensitivity observed in ataxia-telangiectasia cells. This intermediate radiosensitivity observed in ATL2ABR was
similar to the cellular radiosensitivity shown in the fibroblasts of three AOA1 patients and their first-degree relatives in a study by Hannan et al (1994). However, there is still some speculation of IR sensitivity in AOA1 patients and the literature is open on this matter. At a recent A-T conference in September 2003 (Fraser Island, Queensland, AUS) IR sensitivity was debated. While IR sensitivity in AOA1 patients was demonstrated by one group in primary human fibroblasts using colony survival (University of Sussex, UK) it was not seen in other groups including EBV-lymphoblastoid cell lines by our AOA1 group (QIMR, AUS). Further experimental work needs to be carried out on a larger number of AOA1 patients to resolve this discrepancy.

Genetic studies included cloning both APTX splice variants (long APTX and short APTX) into an appropriate inducible vector system for use in ATL2ABR cells. Both forms were successfully cloned into pGem-T easy vector and then the HA-tagged pMep4 vector. However, sequence analysis showed a mutation in the cloned sequence of long APTX in pMep4. However, both L.APTX and S.APTX in pMep4 were transfected into ATL2ABR cells. Induction with cadmium chloride was carried out on both.

Induction was shown to occur between 2-4 hours at a concentration of 5μM of cadmium chloride in S.APTX only. However, no induction could be detected in L.APTX, thus indicating that the random mutation that occurred in L.APTX (due to cloning) resulted in destabilisation of aprataxin as shown by Gueven et al (2004). Induction of short APTX was observed through western blot analysis using the commercial HA antibody for identifying the HA-tagged pMep4. The blots were initially probed with the specific APTX antibody made by Dr. Amanda Kijas in the laboratory. However, only non-specific bands were present in the blots and the
induction was not evident, suggesting that the HA antibody was more specific and sensitive for the detection of short aprataxin. Subsequently, the radiation sensitivity cell survival assay was carried out on these transfected ATL2ABR cells to observe if over-expression of APTX in these potentially APTX deficient cells would alter the radiation sensitivity. No change in the radiation sensitivity was observed in this assay demonstrating that perhaps APTX was not the causative gene for the patient’s disorder and that AOA1 was not the presenting disease. However, this was just speculation and it is possible that aprataxin could correct radiosensitivity in true AOA1 radiosensitive patients.

To further test this hypothesis, dHPLC was carried out on specific exons of the APTX gene to screen for AOA1 mutations in the patient. No mutations were found in any of the screened exons which was supporting evidence that AOA1 was not the clinical condition of the patient.

This data was pooled with other data obtained from various tests carried out on ATL2ABR cells by other laboratory associates. This data is illustrated in table 5.5. Observation of ATM protein levels and ATM kinase activity levels of ATL2ABR indicate that the condition is not A-T. Mre11, Rad51, NBS1 and MRN complex protein levels tested by Western blot analysis and co-immunoprecipitation verify that the condition is not an A-T-like disorder (ATLD). The clinical and laboratory data described in this chapter demonstrates that the ATL2ABR patient was similar to AOA1 more than any of the other autosomal recessive ataxias but has a different underlying cellular and genetic mechanism which contributes to the clinical features of the disease. It was speculated that perhaps the condition was a novel AOA disease entity. Further cellular and genetic testing is required to attempt to elucidate the
mechanisms which are attributing to the clinical symptoms in the patient similar to AOA1.

Further sensitivity studies carried out by associates in the laboratory demonstrated that ATL2ABR cells were hypersensitive to the mitomycin C (MMC) which is a DNA cross-linking agent. This may be a key element in determining the patient’s clinical condition on a cellular level and proteins associated with MMC sensitivity should be investigated further. FANC Proteins are known to be associated with cellular sensitivity to DNA cross-linking agents and they are encoded by genes associated with the rare genetic cancer susceptibility syndrome; Fanconi anaemia (reviewed by Andrea and Gromp, 2002). These proteins are involved in two main cellular mechanisms; DNA repair and S-phase checkpoint cell cycle control. FANC proteins colocalise with proteins such as BRCA1, ATM and MRN in response to DNA damage and are known to be involved in excision repair pathways in association with XPF-XRCC1 protein complex to uncouple crosslink reactions (reviewed by McHugh et al, 2001). S-phase checkpoint control in response to DNA damage involves homologous recombination events at stalled DNA replication forks through activation of proteins such as Mec1 and rad53 as described in chapter 1. The heterodimeric endonuclease Mus81-Eme1 (proposed to be similar to XPF-XRCC heterodimeric unit (Boddy et al, 2000; Interthal et al, 2000)) was also proposed to be involved in replication fork processing and repair. These proteins in association with FANC proteins are important in operating the cellular machinery that contributes to MMC sensitivity. ATL2ABR patient does not display any of the clinical features of Fanconi anaemias patients so the group of FANC proteins are not involved in the condition of ATL2ABR patient. However, investigation of the proteins associated with FANC proteins in ATL2ABR may lead to some of the underlying mechanisms responsible
for a disease clinically similar to AOA1. SNM1 is another putative protein that associated with MMC sensitivity and may also be a candidate responsible for ATL2ABR patients' condition. ATL2ABR cells have been tested for this protein by sequence analysis but no mutation was found. However, further analysis is required to eliminate this protein as a causative protein responsible for ATL2ABR patients' condition. It was postulated that MMC sensitivity may be the key factor for elucidating the genes and proteins associated with the patients' genotype which are responsible for ATL2ABR patients' phenotype. It is possible that a new disease entity may be uncovered adding to the heterogeneous group of autosomal recessive ataxias.
CHAPTER 6

ATAXIA-OCULAR MOTOR APRAXIA (AOA1): A STUDY OF THE SENSITIVITY PHENOTYPE IN AOA1 DEFICIENT AND PROFICIENT CELLS

6.1 Introduction
6.2 Materials and Methods
6.3 Results
6.4 Discussion
Ataxia ocular-motor apraxia (AOA1) is a rare autosomal recessive ataxia characterised by progressive cerebellar ataxia, oculor motor apraxia, choreoathetosis and other neurological and clinical features. This syndrome was first described in 14 patients of ten families by Aicardi et al in 1988 where they described the neurologic features to be similar to AT but the absence of the extraneurologic features of AT such as telangectasia or immunodeficiency. A mean age of onset of 4.7 years was reported in a study of 22 Portugese patients by Barbot et al (2001). A study carried out by Shimazaki et al (2002) reported 5 Japanese patients with early onset AOA from 3 to 12 years with hypoalbuminemia. This was believed to be a separate form of AOA1 known as early-onset ataxia with oculor motor apraxia and hypoalbuminemia (EAOH). However, the causative gene for AOA1 was then mapped and showed that AOA1 and EAOH were the same entity.

Linkage analysis studies demonstrated that AOA1 was mapped to chromosome 9p13 (Moreira et al, 2001) and the causative gene was called APTX (Moreira et al, 2001b; Date et al, 2001). They described the encoded protein of APTX as a histidine-triad protein known as aprataxin. Two major mRNA species encode a long form of aprataxin and a short form of aprataxin as a result of alternative splicing on exon 3. The long form and the short form of aprataxin encode a 342- and a 168-amino-acid protein respectively. The long form of aprataxin is structurally made up of three domains; A PANT domain (PNKP-aprataxin amino terminal); A HIT domain and a zinc-finger carboxy terminal domain. The PANT domain is also known as the FHA domain due to its forkhead protein structure. PNKP is a phosphatase protein that interacts with other proteins (polymeraseβ, DNA ligase III and XRCC1) involved in the SSBR complex (described in chapter 1). This suggests that the long form of aprataxin is involved in
DNA single-strand break repair. The HIT domain is made up of HIT proteins with hydrophobic residues responsible for their distinctive dimeric 10 stranded half barrel structures that form two identical purine nucleotide binding sites (reviewed by Brenner et al, 1999). It was suggested that HIT proteins bind to diadenosine polyphosphate (AP(n)A) complexes (Brenner et al, 1997a) and these function in a proapoptotic tumor suppression pathway and thus have a function in the DNA-damage checkpoint system.

A nuclear localisation signal was proposed to be positioned between the PANT and HIT domain. The zinc-finger carboxy terminal domain is the third structural domain of aprataxin protein. Zinc fingers constitute important DNA and RNA binding domains as well as protein-protein binding domains which are present in many transcription factors (Hartshorne et al, 1986; Vrana et al, 1988). The Cys$_2$/His$_2$ zinc finger has conserved motifs of 28-30 amino acids which are usually present in tandem repeats (Miller et al, 1985; Brown et al, 1985) however both forms of aprataxin contain only a single zinc finger motif. Short aprataxin is structurally composed of the HIT domain at the amino terminal and the zinc-finger domain at the carboxy terminal. It differs from long aprataxin with the absence of the PANT/FHA domain and the nuclear localisation signal. See Figure 6.1 and 6.2 are diagrammatic representations of long and short forms of aprataxin respectively. Other isoforms of aprataxin have been identified in other species such as the macac monkey showing some of the domains previously described. The function of aprataxin is currently under investigation; however, aprataxin is a nuclear protein and was shown to accumulate in the nucleoli and subnucleolar structures by Gueven et al (2004). Aprataxin was shown to interact with DNA SS break repair proteins and components of DNA DSB repair indicating that it plays a role in DNA repair processes (Sano et al, 2004; Gueven et al, 2004)
Several mutations in *APTX* have been associated with the AOA1 phenotype and all of them are within the HIT domain. These mutations vary from missense (617C→T) and nonsense (837G→A) mutations which are base pair substitutions, to frameshift (689insT; 167insT; 318delT) mutations which are base pair insertion and deletion mutations (Date et al, 2001; Moreira et al, 2001b). It was reported that insertion or deletion mutations result in a severe phenotype with childhood onset whereas missense mutations result in a mild phenotype with a relatively late age of onset (Date et al, 2001). These mutations were observed in homozygous/heterozygous alleles in the patients studied.

In this study AOA1 lymphoblastoid cells were obtained from a collaborating group (O. Onodera, Nijgata University, Japan). The cells were encoded LI36, L938 and L939. The mutations on *APTX* attributing to the AOA1 disease phenotype of the patient were known. L938 was homozygous with the same 6AC→T (P206L). Both L939 and L136 were compound heterozygotes; L939 for the mutations 617C→T (P206L amino acid change) and 788T→G (V263G) and L136 for two different frameshift mutations; 689insT and 840delT and the patient from whom the cells were derived had a more severe phenotype than the other two. This could be due to the fact that L938 and L939 cells could have mutated full length aprataxin with residual function, whereas no protein can be found with the two truncating mutations in L136 cells.

In this study immunoprecipitation and Western blot analysis was carried out to detect aprataxin protein levels in these AOA1 patients compared to control cells and ATL2 cells from the chapter 5. These AOA1 cells with mutations in the *APTX* gene might render the protein unstable and so the cells may be deficient in this protein. Although the function of aprataxin is still unclear, it is now evident that it might play a role in the DNA repair process.
The objective of this study was to apply a DNA damaging agent to the AOAl cells which were deficient in aprataxin and form a sensitivity phenotype and then compare this sensitivity to the same genetically altered AOAl cells which were made proficient in aprataxin production. Various DNA damaging agents were initially applied to the AOAl cells. These included ionising radiation, mitomycin C (MMC), methyl methanesulfonate (MMS) and hydrogen peroxide and they were chosen due to their ability to cause DNA single-strand breaks and DNA double strand breaks. The cells were most sensitive to hydrogen peroxide exposure and so this was the DNA damaging agent used throughout the study. The AOAl cells were genetically altered with the cellular transfection of both long and short aprataxin forms cloned into suitable vector systems rendering them proficient in aprataxin protein production. The hydrogen peroxide sensitivity of the AOAl cells was compared to the genetically altered AOAl cells.

Furthermore, a mutation known as 788T→G (V263G amino acid change) which was previously documented by Date et al (2001) and found in the highly conserved HIT region of aprataxin, was also introduced into the APTX expression vector which was transfected into AOAl cells. The mutation was incorporated into long and short aprataxin in their vector systems by site-directed mutagenesis and incorporated into AOAl cells. The hydrogen peroxide sensitivity of these AOAl cells expressing mutant Aprataxin was compared to the Aprataxin deficient and Aprataxin proficient AOAl cells. The mutation K114Q was designed to knock out the nuclear localisation signal in a highly conserved region of aprataxin. These two mutations (V263G and K114Q) are illustrated in the homology map of aprataxin in figure 6.3. Additional deletion mutations for specific regions of aprataxin in EGFP were made and transiently expressed in Hela cells. These mutants were subsequently analysed through
fluorescence microscopy to observe the effects of the mutants on aprataxin protein. The main objective of this study was assess the sensitivity phenotype of AOA1 cells from their genotype through cellular and molecular studies.
**Figure 6.1** Diagrammatic representation of long aprataxin (342-amino acids). The ATG start site in the mRNA for this protein can be seen in exon 1 and the TGA stop site in exon 7. The alternative splicing site is denoted by the blue arrow.

**Figure 6.2** Diagrammatic representation of short aprataxin (168-amino acids). The ATG start site in the mRNA for this protein is in exon 4 and the stop site is as above.
Figure 6.3 Homology protein map of aprataxin demonstrating conservation of the regions between the species. The red box illustrates the position of the mutation V263G in the highly conserved HIT region used in this study.
6.2 MATERIALS AND METHODS

6.2.1 AOA1 cells
EBV-immortalised lymphoblastoid cell lines derived from AOA1 patients were received from Dr. Osamu Onodera, Japan. They were encoded L136, L938 and L939. These were the main AOA1 cell lines used throughout this study. Additional AOA1 cells encoded L990 and L991 were received from Dr. Richard Gatti, UCLA, California, USA. All cells were maintained in RPMI-1640 medium (Gibco™ Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (20IU/20μg per ml) and 10mM L-Glutamine (Both Gibco).

6.2.2 Immunoprecipitations and Western blot analysis of control cells compared to AOA1 cells.

A Specific Antibodies (IgG) used
Specific IgGs were generated in sheep to various domains of aprataxin by Dr. Amanada Kijas (QIMR) using the glutathione S-transferase (GST) gene fusion system (Amersham-Pharmacia Biotech). GST1 IgG was made against the FHA aprataxin domain. GST3 IgG was made for the HIT domain and GST4 IgG was made against the zinc finger domain. It was expected that GST1 IgG should only recognize the long form of aprataxin whereas GST3 and 4 should be specific for both aprataxin isoforms.

B. Immunoprecipitation and Western blotting
The cells were lysed and sonicated as described in chapter 5 to extract the protein from the cells. A protein estimation was carried out using the bio-rad DC protein assay and a
protein solution of 2mg concentration in 700µl volume was made up in lysis buffer (see section I in chapter 5 for detailed protocols). The immunoprecipitation protocol began with a pre-clearing of the lysate. This was done to get rid of the proteins that stick to the beads and protein G non-specifically. 50µl of protein G-agarose beads (1/30 dilution in sephadex 4B (Amersham-Pharmacia Biotech, Denver, Co, USA)) used as a 50% slurry made in protein lysis buffer (see appendices) was added to each of the sample and they were incubated at 4°C for 30mins on a rotating wheel. The samples were then centrifuged at 13,000rpm for 30-60secs. The supernatant was carefully separated from the beads and placed into separate 1.5ml microfuge tubes. The next main step of the immunoprecipitation protocol was the formation of the antibody-antigen complexes. The specific antibody was added to the samples (20µl of fragment 1, 5-10µl of fragment 3 and 2µl of 1/10 diluted day 0 serum as a control). The samples were incubated at 4°C for 3-4 hours on a rotating wheel to allow the antibody-antigen complexes to form. Meanwhile the protein G beads were prepared by washing with lysis buffer three times. 20µl of the protein-G beads in 50% slurry with lysis buffer, was added to the antibody-antigen complexes and they were incubated at 4°C for 1 hour on the rotating wheel. The bead-specific precipitates were removed by centrifugation at 13,000rpm at 4°C for 30seconds. The supernatant was taken off and the beads were washed in lysis buffer. This was repeated 4 times. The residual fluid near the beads on the final wash was pipetted off carefully ensuring not to disturb the beads. Approximately 20µl of loading buffer was added to the beads and they were boiled for 10mins at 100°C in a heating block to linearise the proteins. Subsequent PAGE and western blotting was carried out (as described in chapter 5). The blots were all probed with fragment 4 antibody that should recognise both aprataxin isoforms.
6.2.3 Cloning of APTX into vector systems

Short APTX was cloned into HA-tagged pMep4 vector (pre-made from Dr. Shaun Scott, QIMR) as previously described in chapter 5. Long APTX was cloned into EGFP-C2 (enhanced green fluorescent protein) vector (Invitrogen) by Dr. Nuri Gueven (QIMR) and was used for this study. Both short and long APTX were transfected into all three AOA1 cells by electroporation (the method was described in chapter 6). Stable cell lines were made through specific antibiotic selection. Hygromycin (Gibco) was used to select short APTX in pMep4 vector in the AOA1 cells, whereas Geneticin (Gibco) was used to select long APTX in EGFP vector in the AOA1 cell lines at a concentration of 200|ig/ml. Short APTX protein studies in these AOA1 cells were carried out accordingly as described in chapter 6. Dose and time-dependence of cadmium chloride-induced aprataxin expression was determined using the commercial HA antibody (Roche diagnostics GmbH, Mannheim, Germany). Long APTX protein studies involved western blot analysis (described in chapter 5) of these transfected AOA1 cells using a commercial anti-EGFP antibody (Molecular Probes) to demonstrate its successful transfection into the AOA1 cells. The presence of the vector in AOA1 cells was viewed by fluorescence microscopy.

6.2.4 Site-directed mutagenesis for insertion of 788T→G (V263G) mutation into L.APTX and S.APTX

Site-directed mutagenesis was based on Stratagene QuikChange ® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The protocol used was a modified version (Scott et al; 2002), originally published by Weiner et al. (1994). The protocol was specifically designed to introduce a desired mutation into a designated area (using complementary mutagenesis primers) of double-stranded plasmid DNA.
Two set of primers were designed (Sigma-Genosys, The Woodlands, TX, USA) according to the kit's recommendations for incorporation of the 788T→G (V263G) mutation into long APTX (EGFP) and short APTX (pMep4) and the 340A→C (K114Q) mutation into long APTX (EGFP). See table 6.1 for mutagenic primer sequences. PCR reactions were set up using 75ng, 150ng, 250ng and 500ng of template DNA placed into separate tubes. The following was added to each tube; 125ng of each primer, 10mM dNTPs, 2.5U/µl of PFU turbo and supplied buffer (Stratagene) and double-distilled water to a final volume of 50µl. The PCR conditions were as follows; denaturation at 95°C for 5 min, followed by 20 cycles of denaturation at 95°C for 30 secs, annealing at 59°C for 30 secs and extension at 72°C for 2mins/kb length of plasmid and insert.

The whole PCR reaction was then incubated with 20U of Dpn 1 (New England Biolabs Inc, Beverly, MA, USA) at 37°C for 3 hours. Dpn 1 enzyme acts by digesting the methylated nonmutated parental DNA template from the reaction, leaving the mutated DNA for selection of true mutant colonies. An ethanol precipitation was carried out on the digested parental DNA and half of the resuspension volume was transformed into dH5α™ competent cells (Invitrogen, CA, USA), selected by ampicillin (Gibco) resistance for short APTX in pMep4 and kanamycin (Gibco) resistance for long APTX in EGFP. The plasmid DNA was extracted using a Qiaprep® spin miniprep kit (Qiagen GmbH, Hilden, Germany). The details of which are described in chapter 5. The presence of the desired mutation was verified by sequence analysis. The correct insertion of the mutation was checked by sequence analysis (described in chapter 4).

Large quantities of the sequence verified plasmids were obtaining using bulk cultures and a Perfectprep® plasmid midi prep kit (Eppendorf GmbH, Hamburg, Germany).

These DNA samples were subsequently transfected into all three AOA1 cell lines.
through electroporation as described in chapter 5. Stable transfected cell lines were made by specific antibiotic selection using hygromycin with mutated \textit{APTX} in pMep4 and geneticin with mutated \textit{APTX} in the EGFP vector.

\textbf{Table 6.1:} Primer Sequences for insertion of 788T→G (V263G) and 340A→C (K114Q) mutation into \textit{APTX} through site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' – 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{APTX}-788T→G</td>
<td>5'GCCATTCCGAGTATGAGCCATGTACATCTTCATGGGATCAGCCAGGAT TTTGATTCC'3</td>
</tr>
<tr>
<td>Forward Primer</td>
<td></td>
</tr>
<tr>
<td>\textit{APTX}-788T→G</td>
<td>5'GCAAGGAGAATCAAATCCTGGCTAGATCCCATGAAGATGTACATGGCT CATACTCGGAATGGGC'3</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>\textit{APTX}-340A→C</td>
<td>5'GGCCTGGAAACACACACAGGCAGAGAAAGAGATCAGGTC'3</td>
</tr>
<tr>
<td>Forward Primer</td>
<td></td>
</tr>
</tbody>
</table>
| \textit{APTX}-340A→C   | 5'GCCTGATCTCTTTTCTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
same initial volume of media (5mls) in new flasks. Hydrogen peroxide damage occurs instantly in cells and so the cells were only exposed for 30 minutes. All of the other agents caused damage at a slower rate and could be incubated with the cells. After exposure, cells were incubated for 48 hours at 37°C and 5% CO₂. At 48 hours the trypan blue viability test was carried out as described in chapter 5.

6.2.6 Complementation studies of the sensitivity of transfected cells versus non-transfected cells to hydrogen peroxide.

The cell survival assay using hydrogen peroxide as the DNA damaging agent was used to assess the sensitivity of AOA1 cells transfected with both long and short APTX in contrast to their sensitivity phenotype without transfection. Short APTX in pMep4 transfected cells were induced with 5mM cadmium chloride for 12 hours at 37°C.

Figure 6.4 Diagrammatic representation of the enhanced green fluorescent Vector, EGFP-C2 used with long APTX.
6.2.7 Transient expression of mutant APTX-EGFP expressed in HeLa cells for fluorescence microscopical analysis

Primers were designed to clone specific regions of APTX (Sigma-Genosys). Forward primers were made with the incorporation of an XhoI restriction site and one reverse primer was made with the incorporation of a BamHI restriction site for use with all forward primers. The primer sequences, codes and specific APTX region are illustrated in Table 6.2. Cloning was carried out by Dr. Nuri Gueven (QIMR) using long APTX in EGFP vector as the template DNA (described in chapter 5). The DNA extracted from the recombinant clones was sequence verified. HeLa cells were plated onto coverslips which were aseptically placed into wells of 6-well plates (Falcon, St.Louis, MO, USA) in 100μl volume. They were incubated overnight at 37°C and 5% CO2. Transient transfection was carried out using the protocol provided with lipofectamine™2000 (Invitrogen). 0.4μg of mutant DNA was diluted in 25μl of serum-free RPMI-1640 (Gibco) medium. 1μl of of lipofectamine 2000 was diluted also in 25μl of serum-free medium and incubated at room temperature for 5 minutes. The diluted DNA was then mixed with the diluted lipofectamine, mixed gently and incubated at room temperature for 20minutes to allow the DNA-lipofectamine complexes to form. The 50μl volume of DNA-lipofectamine complexes was added to a well of HeLa cells and mixed gently. The cells were incubated at 37°C with 5% CO2 for 24 hours. 2mls of RPMI-1640 supplemented with 10% foetal calf serum and 10mM of L-Glutamine was added to each of the wells containing the transfected cells. The cells were incubated for a further 24 hours at 37°C. The coverslips containing the transfected cells were carefully transferred from the wells of the 6-well plate and inverted onto a glass slide for fluorescence microscopical analysis.
Table 6.2 Primer sequences for specific APTX mutations used with long APTX in EGFP.

<table>
<thead>
<tr>
<th>Code</th>
<th>Primer Sequence</th>
<th>Specific Regions of APTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-LA</td>
<td>Full length clone</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
<tr>
<td>E-LA V263G</td>
<td>Previous Mutated long</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
<tr>
<td>E-LA</td>
<td>Mut 1-F</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
<tr>
<td>E-LA</td>
<td>Mut 2-F</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
<tr>
<td>E-LA</td>
<td>Mut 3-F</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
<tr>
<td>E-LA</td>
<td>Mut 4-F</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
<tr>
<td>E-LA K114Q</td>
<td>Previous mutated long</td>
<td><img src="Diagram.png" alt="Diagram" /></td>
</tr>
</tbody>
</table>
6.3 RESULTS

6.3.1 Sensitivity of AOAl cells to various DNA damaging agents.

To determine whether AOAl cells like those from patients with A-T, were hypersensitive to DNA damaging agents, they were exposed to several different damaging agents. The AOAl cells showed no hyper-sensitivity to ionising radiation (Figure 6.5) and were similar to the control cell line (C3ABR) in contrast to the radiation sensitivity of the A-T cell line (AT1ABR). The AOAl cells also showed no hypersensitivity to the DNA crosslinking agent; mitomycin C (MMC) (Figure 6.6). They showed an overlap in sensitivity to both the control and A-T cell line. However, enhanced sensitivity to methyl methanesulfonate (MMS) was observed in the AOAl cell lines when compared to both the control and A-T cells (Figure 6.7). The AOAl cells were also exposed to hydrogen peroxide which was another agent chosen because it causes single-strand breaks in DNA. The AOAl cells displayed a three-fold increased sensitivity to the control cells with 0.5 and 1mM of hydrogen peroxide (Figure 6.8) demonstrating that the AOAl cells were more sensitive to hydrogen peroxide compared to MMS.

6.3.2 Immunoprecipitation (IP) and Western blotting of AOAl cells.

Aprataxin protein detection was compared in AOAl cells (L136, L938 and L939) versus control (C3ABR), A-T (AT1ABR) and ATL2ABR cells through Western blot analysis and probed with fragment 4 antibody (detects the zinc finger domain of aprataxin) so that both long and short aprataxin could be observed (figure 6.9). Immunoprecipitation (more specific protein detection method) was carried out on AOAl cells (L938 and L939) versus control (C3ABR) with specific antibodies against
specific fragments of aprataxin. Fragment 1 (recognises the FHA domain) was specific for long aprataxin and fragment 3 (recognises the HIT domain) was specific for both isoforms. IP of L938 AOA1 and control cells with week 0 serum was added as a negative control (figure 6.10). The unbound proteins from the IP were analysed through PAGE and western blotting and probing with fragment 4 antibody. The unbound proteins were analysed to show that although aprataxin is detected in the AOA1 cell lines from the immunoprecipitation which specifically targets the protein in figure 6.10, there are lesser amounts present than in the control (C3ABR) cells. Figure 6.11 shows a band present in the C3ABR control cells for long and short aprataxin. No bands are present in the AOA1 cells (L938 and L939), indicating that the immunoprecipitation picked up all of the aprataxin protein from the AOA1 cell with none remaining in contrast to the remaining quantities in the control cells (C3ABR).
Figure 6.5 Sensitivity of AOAl cell lines to ionising radiation (0-4Gy) compared to a control (C3ABR) and AT (AT1ABR) cell line.

Figure 6.6 Sensitivity of AOAl cells to MMC (0-6mM) compared to a control (C3ABR) and AT (AT1ABR) cell line.
Figure 6.7 Sensitivity of AOAl cells to MMS (0-0.1mM) compared to control (C3ABR) and AT (AT2ABR) cell lines.

Figure 6.8 Sensitivity of all AOAl cell lines to hydrogen peroxide (0-1mM) in contrast to control (C3ABR) and AT (AT2ABR) cell lines.
Figure 6.9 Western blot analysis of control (C3ABR), A-T (AT1ABR), AOA1 (L136, L938 and L939) and patient cells (ATL2ABR). The western blot was probed with fragment 4 antibody to specifically detect the two forms of aprataxin. This figure demonstrates the reduction/absence of long (45kDa) and short (25kDa) aprataxin in the AOA1 cells compared to the control, A-T and ATL2ABR cells.
**Figure 6.10** Western blot analysis of immunoprecipitation of control (C3ABR) cells versus AOA1 cells (L938 and L939) with specific APTX antibodies (fragment 1, fragment 3 and week 0 serum) to show two isoforms of aprataxin. Fragment 1 antibody was specific for the FHA domain present only in the long form of aprataxin and fragment 3 was specific for the HIT domain present in both isoforms. In contrast the week 0 serum was used as a negative control and did not detect either of the isoforms of aprataxin. The recombinant yeast APTX protein was added to the western blot as a positive control to compare the bands of each aprataxin isoform. The western blot was also probed fragment 4 antibody to detect the two aprataxin isoforms.
**Figure 6.11** Western blot analysis of unbound aprataxin protein from immunoprecipitation of control (C3ABR) versus AOA1 cells (L938 and L939). This figure demonstrates that there is remaining aprataxin protein in the controls compared to the AOA1 cells after the immunoprecipitations, and thus lower levels of aprataxin in AOA1 patients. The recombinant yeast protein was a positive control for comparing the bands of both aprataxin isoforms.
6.3.3 Expression of EGFP (long APTX) and HA-pMep4 (short APTX) transfected into L.938 and L.939 AOA1 cells lines.

Successful transfection of long APTX in EGFP-C2 vector was confirmed by fluorescence microscopy. The enhanced green fluorescence protein vector showed fluorescence (EGFP) in the nucleus of the AOA1 cells. Immunoprecipitation of EGFP from the AOA1 cells with EGFP antibody was carried out to confirm that the long form cDNA in the vector got expressed in the AOA1 cells. This demonstrated the presence of long aprataxin protein with the EGFP tag in contrast to the AOA1 cells with the V263G AOA1 mutation (figure 6.12). Successful transfection of short APTX in HA-tagged pMep4 was demonstrated by induction of the vector system with cadmium chloride concentrations over various time points (figures 6.13 and 6.14).

**Figure 6.12** Immunoprecipitation of transfected AOA1 cells with L.APTX in EGFP vector and mutated (V263G) L.APTX in EGFP vector with EGFP antibody. Blot probed with specific rabbit APTX antibody.
6.3.3 Induction of $SAPTX$ in pMep4 in ATL2 cells

Expression of $SAPTX$ in pMep4 was induced in the transfected AOAl (L938 and L939) cells with 1-5μM of Cadmium chloride after 16 hours. The induction appeared concentration-dependent (See figure 5.13). No band was observed in the control (0) concentration in contrast to the appearance of a band at the first concentration of 1μM. This band was stronger with increasing concentration and displaying a band two-fold bigger with double the concentrations between 1-2.5μM and 2.5-5μM. Therefore 5μM of Cadmium Chloride was used to induce the transfected cells for the subsequent time-point induction experiment. In this experiment, it was demonstrated in L938 and L939 cells that aprataxin was induced at 2 hours after addition of 5μM Cadmium chloride and expression increased further between 2-4 hours. Induction increased slightly at each time-point between 4-12 hours (the latter showing the highest induction). This is illustrated in figure 6.14. These results were the same as the short form of aprataxin expression results for the ATL2ABR cells in chapter 5.
**Figure 6.13** Western blot analysis of transfected AO1 cells (L938-T and L939-T) with short *APTX* in HA-tagged pMep4 vector induced with various concentrations of cadmium chloride (0-5μM). Blot probed with specific HA antibody.

<table>
<thead>
<tr>
<th>Cadmium Chloride (μM)</th>
<th>0</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>L938</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L939</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6.14** Western blot analysis of transfected AO1 cells (L938-T and L939-T) with short *APTX* in HA-tagged pMep4 induced with 5μM cadmium chloride at different time points. Blot probed with specific HA antibody.

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.938-T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.939-T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3.5 Insertion of 788T→G (V263G) mutation into L.APTX and S.APTX by site-directed mutagenesis.

Recombinant clones with the inserted mutation 788T→G were verified through sequence analysis. Figure 6.15 and 6.16 demonstrates the sequences obtained from mutated clones of short APTX in pMep4 vector and long APTX in EGFP vector. Forward and reverse sequences of both short and long APTX were analysed.

**Figure 6.15** Verified insertion of 788T→G (V263G) mutation into short APTX through sequence analysis. Forward sequence demonstrated.
Figure 6.16 Verified insertion of 788T→G (V263G) mutation into long \textit{APTX} through sequence analysis. Reverse sequence demonstrated.

\[
\text{Reverse 788T→G primer Sequence: 5'}GCAAGGAGAATCAAAATCCTGGCTGATC(\text{CATGAAGATGTACATGGCT')}3
\]

6.3.6 Complementation studies through cell sensitivity to hydrogen peroxide.

Sensitivity of AOAl (L939) cells transfected with long \textit{APTX} in EGFP was compared to AOAl cells transfected with the mutated (788T→G/V263G) long \textit{APTX} in EGFP. EGFP vector without insert was used as control in AOAl (L939) and control (C2ABR) cells (figure 6.17). The control EGFP vector in L939 cells and the mutated long \textit{APTX} in L939 cells showed sensitivity to hydrogen peroxide. However, the long \textit{APTX} in L939 cells demonstrated no sensitivity to hydrogen peroxide and were similar to the control (C2ABR) cells. This data demonstrated correction of the sensitivity phenotype in AOAl cells through the insertion of long \textit{APTX} into these cells.

Sensitivity of short \textit{APTX} in pMep4 transfected in AOAl cells both induced (with 5mM of cadmium chloride) and uninduced was compared with induced mutated (788T→G/V263G) short \textit{APTX} in pMep4 transfected in AOAl cells, control (C2ABR) cells and control cells transfected with short \textit{APTX} in pMep4 induced with cadmium.
chloride (figure 6.18). The uninduced short $APTX$ transfected L939 cells showed similar sensitivity to the induced mutated short $APTX$ L939 cells. No difference in sensitivity was observed between the transfected and non-transfected control (C2ABR) cells. The induced short $APTX$ in L939 cells showed no sensitivity to hydrogen peroxide and were also similar to the control cells. This data demonstrates correction of the sensitivity phenotype in AOA1 cells through insertion of short $APTX$ into these cells.

6.3.7 Fluorescent staining of long $APTX$ in EGFP

The knockout mutations for specific regions of aprataxin (described in table 6.2) were compared with non-mutated aprataxin by fluorescence microscopy. The V263G and K114Q mutations in the long form of aprataxin in EGFP were also analysed. All images displayed fragmented cellular debris, indicating the protein was destabilised due to the mutations (figure 6.19), with the exception of the K114Q mutation which was designed to knockout the first nuclear localisation signal. In these cells nuclear staining was still present; however extranuclear, cytoplasmic staining was also observed.
Figure 6.17 Long APTX complementation data: Sensitivity of long APTX in L939 cells (L939-EGFP-L) compared to mutated long APTX in L939 cells (L939-EGFP-V), control vector in L939 cells (L939-EGFP-C) and control (C2ABR) cells.
**Figure 6.18** Short *APTX* complementation data: Sensitivity of induced and uninduced short *APTX* in L939 cells (L939-SA) compared to induced mutated short *APTX* in L939 cells (L939-V263G), induced short *APTX* in control cells (C2ABR-SA) and control cells (C2ABR).

![Graph showing sensitivity of AOA1 and control cells transfected with short APTX in pMep4.](graph.png)
Figure 6.19 Cellular localisation of aprataxin depicted by A. immunostaining of normal human fibroblasts (NFF) and AOA1 cells (FG4003) stained for aprataxin with fragment 4 antibody. B. EGFP-aprataxin construct that was transiently transfected in NFF and HeLa cells
**Figure 6.20** Fluorescent microscopy of Long *APTX* in EGFP in contrast to deletion mutations made in long *APTX* in EGFP (E-LA). Two pictures were taken for each.

<table>
<thead>
<tr>
<th></th>
<th>Picture 1</th>
<th>Picture 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Long APTX in EGFP</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>B.</td>
<td>Long APTX in EGFP with V263G mutation</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>C.</td>
<td>Long APTX in EGFP with Mut 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 6.20

D. Long APTX in EGFP with Mut 2

E. Long APTX in EGFP with Mut 3

F. Long APTX in EGFP with Mut 4
Figure 6.20

G. Long APTX in EGFP with NLS mutation
6.4 DISCUSSION

Early onset ataxia with oculomotor apraxia (AOA1) is a human genetic disorder which has been mapped to chromosome 9p13 (Moreira et al, 2001; Date et al, 2001). The defective gene is known as APTX and it was predicted to encode 342- and a 168-amino acid proteins through alternative splicing. These splice variants were termed long and short forms of aprataxin respectively. In this study, the long and short forms of aprataxin were shown to be expressed as a 45kDa (Gueven et al, 2004) and a 25kDa protein respectively. The long form of aprataxin was shown to be expressed as a 37kDa by Sano et al (2004). Discrepancies in size of long aprataxin between these groups may be due to many factors such as those between methods, cells etc. It is also possible that the existence of other isoforms mimicking the long form of aprataxin may be a factor. The short form of aprataxin was suggested to be 25kDa by Sano et al (2004).

The three main AOA1 cell lines used for this study were encoded L136, L938 and L939. L136 cells were the hardest cells to grow between the AOA1 cell lines. They required a higher concentration of foetal calf serum for their maintenance and growth and their proliferative capacity became weaker over a period of time. These cells were derived from an AOA1 patient heterozygous for frameshift mutations (689insT/840delT) which results in a more severe phenotype and thus might explain the condition of the cells. These cells were only used for the initial cell survival experiments to various DNA damaging agents. In contrast, L938 and L939 AOA1 cells were more stable and were used for the remaining experiments demonstrated in this study.

Analysis of these AOA1 cells by immunoblotting (Figure 6.9) demonstrated the absence of both long and short forms of aprataxin in AOA1 cells in contrast to the presence of aprataxin in control (C3ABR), AT (AT1ABR) and ATL2 patient (Chapter
5). However, immunoprecipitation of AOAl cells (L938 and L939) and control cells (C3ABR) with specific antibodies raised in sheep for specific regions of aprataxin show the same levels of aprataxin (both long and short) in contrast to the negative control (week 0 serum). All antibodies were made by Dr. Amanda Kijas (QIMR). The unbound proteins from this immunoprecipitation were analysed by immunoblotting and the difference in levels of aprataxin between AOAl cells and control cells was demonstrated. This data demonstrated that AOAl cells were not completely depleted of aprataxin protein levels but there was a lower level of both long and short aprataxin in contrast to control cells suggesting that the presence of the AOAl missense mutations in these cells, L938 (homozygous for P206L mutation) and L939 (heterozygous for P206L/V263G) destabilised aprataxin protein production.

Long and short aprataxin were cloned into EGFP and HA-pMep4 respectively and transfected into AOAl (L938 and L939) cells. The presence of EGFP in transfected cells was viewed by fluorescence microscopy. Although fluorescence could be detected, the cells (lymphoblastoid cell lines) did not clearly show this and the captured images were vague and not included in this study. To counteract this, immunoprecipitation of the transfected AOAl cells with EGFP antibody was carried out. Western blot analysis of this immunoprecipitation demonstrated the low levels of long aprataxin in the AOAl cells (as described above). In contrast, a mutated (V263G) version of the EGFP-tagged long form of Aprataxin was not detectable, which supported the hypothesis that mutations in APTX destabilised the Aprataxin protein.

Short aprataxin in HA-tagged pMep4 was detected through western blot analysis of transfected cells induced with 5mM of cadmium chloride for 4-12 hours. The anti-HA antibody was used for this HA-tagged system. The specific APTX antibodies used in the above immunoprecipitation and immunoblotting experiments were not available at
the time these experiments were conducted. These antibodies were made over a 6 month period by Dr. Amanda Kijas (QIMR) involving various fusion protein injection and blood extractions from sheep. The antibodies were then purified for subsequent use. Some of the antibodies that were made and purified at an earlier stage, was used in initial experiments on induced transfected cells and compared to the use of the commercial HA-antibody. Aprataxin was only detected with the HA-antibody. The specific aprataxin antibody appeared to be less sensitive and resulted in non-specific banding. Thus, the high affinity HA antibody was used for the induced-transfection experiments due to its high sensitivity (as it can detect HA-tagged aprataxin in less then 10μg total cell extract) and specificity. The presence of the short form of aprataxin is disputed to date between the aprataxin research groups. Sano et al (2004) detected a short form at 25Kb similar to the short form detected in this study, however this is only a very faint signal which can be disputed. However, Caldecott et al (university of Sussex) and Taylor et al (university of Birmingham) could not detect a short form at all. It appears that the long form of aprataxin is the dominant form and results appear to be consistent with these between the laboratories. Also from the northern blot data shown in the paper by Date et al (2001) which showed the two mRNA species, that the upper larger band (thought to represent the long form) was the most prominent between the two species. From these date, it is evident that further studies showing the short form of aprataxin need to be carried out.

The predicted function of aprataxin is as a protein involved in the single strand break repair (Moreira et al, 2001a; Date et al, 2001) because of the functional domains in aprataxin protein structure. The initial sensitivity studies of AOA1 cells in contrast to control cells demonstrated that AOA1 cells were not sensitive to ionising radiation like A-T or MMC, in contrast to the MMC sensitivity of the ATL2 patient. AOA1 cells
showed sensitivity to MMS and hydrogen peroxide and these agents are known to cause single strand breaks in DNA (Solveig Walles and Erixon, 1984; Ward et al, 1987) supporting the hypothesis that aprataxin is involved in single strand break repair processes.

Both long and short aprataxin were transfected separately into AOAl cells rendering them “proficient” in each form of aprataxin. It was demonstrated that the sensitivity of these cells to hydrogen peroxide was reversed to a similar level as the control. Furthermore, decreased genomic instability was observed in these cells through cytogenetic analysis by Dr. Phil Chen (QIMR). This data was demonstrated in the paper by Gueven et al (2004). Transfected cells with the inserted mutation 788T→G (V263G) in long and short APTX showed no change in hydrogen peroxide sensitivity.

Aprataxin was shown to be a nuclear protein, present in the nucleus and nucleolus (Gueven et al, 2004) using EGFP-tagged aprataxin. Long aprataxin was cloned into EGFP vector. The advantage of this system was that fluorescence microscopy demonstrated the localisation of aprataxin. A putative nuclear localisation signal in aprataxin is found between the FHA and HIT domains and was suggested to be the most significant for the localisation of long APTX in the nucleus (Sano et al, 2004). Two additional nuclear localisation signals were proposed in this study. One further downstream from the first nuclear localisation signal between the FHA and HIT domains, and the other in the zinc-finger domain. With site directed mutagenesis, the mutation K114Q was inserted to knock out the function of this nuclear localisation signal. Mild extra-nuclear/cytoplasmic staining was observed in the cells containing this mutation however aprataxin was still localised in the nucleus. These results indicated that the mutation did affect the nuclear localisation causing the cytoplasmic staining but it disagrees with the hypothesis that this first nuclear localisation signal is
essential for localisation of long aprataxin in the nucleus. Furthermore, this data reinforced the hypothesis that other nuclear localisation signals are present in aprataxin. Additional fluorescence microscopical studies demonstrated the condition of aprataxin in response to the various deletion mutations made. We had to expose the cells 10 times longer under the microscope to see the faint residual signal. It is evident that these mutants caused destabilisation of aprataxin. This supported the previous findings from the V263G mutation in both the immunoprecipitation and cell survival experiments.

This data demonstrated that AOA1 mutations cause the destabilisation and loss of aprataxin function of both forms (long and short aprataxin) and that sensitivity of AOA1 cells to agents causing single strand break repair can be corrected with the insertion of \textit{APTX} into the cells implicating the role of aprataxin in single strand break repair.

This hypothesis was consolidated with evidence that aprataxin interacts with XRCC1 (Sano et al, 2004) and PARP-1 (Gueven et al, 2004). Interaction of the FHA domain (specific for long aprataxin) with XRCC1 was observed while interaction of the FHA and also the HIT region (specific for long and short aprataxin) with PARP-1 was detected. XRCC1 binds to PARP-1 as part of the base excision repair complex (Masson et al, 1998). They hypothesised that PARP is involved in the detection of DNA damage and possibly recruits XRCC1 and its binding partners. XRCC1 interacts with APE1 (Vidal et al, 2001); DNA polymerase β (Caldecott et al, 1996; Kubota et al, 1996); DNA polynucleotide kinase (Whitehouse et al, 2001) and DNA ligase III (Caldecott et al, 1994) and they are involved in base excision repair processes (Masson et al, 1998). Thus, interaction of the HIT region with PARP-1 implicates both long and short aprataxin irrespective of the specific FHA region of long \textit{APTX} (interacting with
XRCC1) in the single strand break repair process. The interaction of aprataxin with these single strand break repair proteins describes a possible underlying cellular pathway in response to DNA damage in AOA1 patients. However, these findings are recent and further experimental analysis is required to consolidate these observations and hypotheses.
CHAPTER 7

GENERAL DISCUSSION
This thesis provides evidence that the biology of a cell involves a complex network of cellular and molecular mechanisms employed to maintain the integrity of the genome which is vital for cellular and organism homeostasis. Genomic integrity can become compromised due to many factors such as hereditary content or spontaneous alterations which can arise from endogenous cellular processes and/or exogenous factors causing distinct genetic defects. Although disease, whether it be a malignant or non-malignant disorder may be associated with distinct genetic defects in specific genes, it is the cellular processes of replication, mitosis and repair that respond to these genetic alterations (either inherited or acquired). These processes involve a complex network of biochemical events for activating and deactivating specific proteins to respond to the various genetic alterations depending on the extent of the type of DNA damage and the fate of the cell is decided.

The overall objective of this thesis was to investigate the underlying cellular and molecular mechanisms that occur in response to genetic alterations in a variety of disorders with a genetic component and to increase our knowledge of the aetiology and pathogenesis of these diseases. This was performed by manipulating the cell cycle events by exploring mutagen sensitivity in the cells from these different patient groups. Thus the known disruptive targeted effect of the mutagen was the key for this analysis. Mutagens begin their action by forming DNA adducts and these are a result of the covalent binding of the all or part of the mutagen to the nucleotide base in DNA. Various types of damage to the double helix are formed including single strand breaks, double strand breaks, chemically modified bases, strand crosslinks, base-pairing mismatches etc. Ionising radiation which mainly forms double strand breaks was the first mutagen investigated in breast (Chapter 2) and prostate (Chapter 3) cancer patients and the autosomally recessive genomic-instability condition A-T
(Chapter 4). Hyper-sensitivity to radiation and increased chromosomal aberrations are features of A-T demonstrated in the existing literature. Furthermore, A-T has been linked to many cancers including breast and prostate cancer and thus, levels of elevated sensitivity and increased chromosomal aberrations were expected in these malignancies also. Swift et al (1991) carried out an epidemiological study on 161 families with A-T and demonstrated that a proportion of these patients had developed various cancers including breast and prostate cancer. Particular emphasis has been placed on the association of ATM heterozygosity and the occurrence of breast cancer. Easton et al (1994) demonstrated that approx. 3.8% of breast cancer patients are ATM carriers, but other studies indicate that this figure may be as high as 13% (Stankovic et al, 1998). Prostate cancer has also been associated with A-T but to a lesser extent. Hall et al (1998) showed that a small proportion of prostate cancer patients undergoing radiotherapy who experience radiotherapy treatment late effects were A-T heterozygotes and more recently Cesaretti et al (2004) also showed an association with ATM in 16/37 prostate patients who respond to radiotherapy. Occurrence of breast cancer and prostate cancer have also been associated in the literature. Ford et al (1998) proposed that BRCA1 and BRCA2 carriers have a 6% and 6-12% risk of developing prostate cancer respectively. More recently, Dong et al (2003) identified mutations in CHK2 in prostate cancer and furthermore proposed the association of BRCA1 and BRCA2 mutations with prostate cancer risk.

In chapter 2 and 3, elevated levels of G2 chromosomal damage induced by IR, similar to A-T levels (in the literature) were demonstrated using the G2 chromosomal radiosensitivity assay. This demonstrates that similar cellular mechanisms may operate in A-T and in breast and prostate cancer formation. The key molecules that may be implicated are ATM and P53. ATM is a transducer protein that is
phosphorylated after DNA damage. It is sensed and it activates effector proteins
downstream in the cellular cascade to halt the cell cycle in the G2/M or G1/s
checkpoint to allow enough time to decide the fate of the cell. The damage is either
repaired by one of the complex repair systems described in section 1.6 (chapter 1) or
apoptosis is induced as described in section 1.7 (chapter 1). Figure 7.1 indicates a
diagrammatic representation of the possible outcomes of a cell post exposure to DNA
damaging agents.

Alternatively, the genetic alteration may allow the cell cycle checkpoint to be
traversed and the alteration in the cell progresses in the cell cycle. The damage may
be propagated in some cases, or alternatively delayed cell death or mitotic death can
occur (as described in section 1.7, chapter 1). In Chapter 3, an inverse relationship
between spindle defects (which are associated with mitotic death) and G2
radiosensitivity was demonstrated, indicating that apoptosis is the immediate death
mechanism for IR damaged radiosensitive cells in contrast to mitotic death being
associated with IR damaged radioresistant cells.

P53 was the first tumour suppressor gene to be linked to apoptosis (Vogelstein, 2000)
and it plays a vital role in the control of apoptosis and cancer development. P53 is
recognised as the most commonly mutated gene in human malignancies (Lane, 1994).
When DNA damage occurs, it can trigger cell cycle arrest to allow repair processes to
occur or trigger apoptosis to prevent tumour formation. Thus, defects in P53 can aid
in the carcinogenic process. A review by Kurz and Miller (2004) demonstrates the
effects of IR along with other DNA damaging agents on the activation and
phosphorylation of ATM and P53. It shows that DNA damage induced by ionising
radiation exposure stimulates the kinase activity of ATM, P53 protein is accumulated
dependent on ATM. Figure 7.2 taken from Kurz and Miller (2004) demonstrates the
complex cellular processes that occur when ATM is activated in response to double
strand breaks (from IR) and other forms of DNA damage. The cellular and molecular
processes that are activated or deactivated to respond to the DNA damage are
highlighted. The processes of cell cycle checkpoint control, DNA repair, apoptosis,
and transcription control which decide the fate of the cell in response to the genetic
alteration induced by mutagens are indicated in this diagram.

Subsequent to these radiobiological studies, other conditions in the autosomal
recessively inherited ataxias were investigated, using radiosensitivity and
chromosomal instability as biological endpoints. The patient encoded ATL2ABR
described in chapter 5 which had similar clinical features of A-T but no telangectasia
and intermediate radiosensitivity led to speculation that the presenting disease was
Ataxia-oculomotor apraxia 1 patients. However, through subsequent cellular
sensitivity studies with the cloned APTX gene inserted into the cells, it was shown
that loss of aprataxin function was not responsibe for the clinical phenotype. It was
later shown to have sensitivity to mitomycin C which causes DNA damage by
forming strand cross-links and furthermore not similar to the mechanisms that operate
in A-T as ATM phosphorylation has been shown to be not significant for mitomycin
C DNA damage (Kurz and Lees-Miller, 2004). Furthermore, subsequent studies on
cells from specific AOA1 patients showed sensitivity to Methyl methanesulfonate
(which is an alkylating agent) and hydrogen peroxide (which forms DNA damage
from reactive oxygen species). These agents were shown to form single strand
breaks (Solveig Walles et al, 1984; Ward et al, 1987) in DNA, which suggests some
of the underlying mechanisms of this AOA1 (and a function of aprataxin) that are
comparable to A-T. Furthermore, Cantoni et al (1989) demonstrated that
radiosensitive A-T cells are not sensitive to hydrogen peroxide. Following from this,
studies have shown that single strand break repair proteins interact with specific
domains of aprataxin (Hirano et al, 2003; Gueven et al, 2004). A study carried out by
Clements et al (2004) demonstrated the cellular and molecular differences of A-T and
AOA1. ATM was absent in A-T and present in AOA1, similarly APTX was absent in
AOA1 and present in A-T. Reduced/delayed phosphorylation of P53 and CHK2 was
also observed in AOA1 compared to A-T. The AOA1 study in chapter 6, further
demonstrated that mutations in the APTX gene responsible for the symptoms of
AOA1 may be attributable to the loss of aprataxin function due to either, a diminished
activity of the proteins, destabilisation of the protein or mislocalisation of the protein.
Cloning of the APTX gene in the deficient AOA1 cells, making them APTX
proficient corrected aprataxin function (possibly in single strand break repair) and
corrected the hydrogen peroxide sensitivity phenotype of the cells. Although the
functions of ATM and APTX appear to be different in the underlying cellular and
molecular pathways in response to DNA damage, the cell operates within its specific
pathways to cope with the genetic alterations induced. ATM is a central molecule
operating the cellular machinery in response to DNA damage and hydrogen peroxide
has been shown to stimulate ATM kinase activity (Chen et al, 2003) implicating its
important role in cellular processes. ATR which is protein associated with ATM, as
they are from the phosphatidylinositol 3-kinase family, has been shown to be
associated with single strand breaks and so may have an important role with
aprataxin. However, this area is relatively novel and additional studies must be
carried out to elucidate this in more detail.

In conclusion, this thesis demonstrates that the use of DNA damage models are
powerful tools for scientists to elucidate the underlying molecular and cellular
processes occurring in specific clinical conditions in response to genetic alterations.
Furthermore, they can increase our knowledge in the aetiology and pathogenesis of the disease as well as improving understanding, prevention, diagnosis or treatment of the disease.
Figure 7.1 Illustration of the fate of the cell post exposure to exogenous DNA damaging agents.
Figure 7.2: Kurz and Lees-Miller, 2004

A model for activation of ATM by double strand breaks and other forms of DNA damage. The MRN complex is proposed to detect DNA damage and signal ATM through phosphorylation. ATM signals a cascade of signals to respond to the DNA damage and the fate of the cell is decided through the mechanisms.
MOLECULAR BIOLOGY REAGENTS

Protein Cell lysis Buffer
- 50mM Tris pH 7.4
- 150mM NaCl
- 2mM EDTA
- 2mM EGTA
- 25mM β-glycerol-phosphate
- 0.2% Triton X-100
- 0.3% NP-40

Added before use
- Protease inhibitors
  - 1M DTT
  - 1M PMSF
- 25mM NaF
- 0.1mM NaVa

10X TBS-T
- 24.2g Tris
- 80g NaCl
- Dilute in 1 litre of deionised water and PH to &.6 with HCl
**1X TBS-T**
- 2.42g/l Tris
- 8.0g/l NaCl$\_2$
- Dilute in 1 litre of deionised water and PH to 7.6 with HCl
- Add 0.1% tween

**10X High molecular weight transfer buffer**
- 60g/l Tris
- 30g/l Glycine
- 36ml of 10% SDS
- Dilute in 1 litre of deionised water

**1X High molecular weight transfer buffer**
- 100mls of 10X high molecular weight buffer
- 200mls of methanol
- 700mls of deionised water

**50X TAE buffer**
- 242g/l Tris base
- 37.2g/l Na$_2$EDTA
- 57ml acetic acid
- Diluted with deionised water up to 1 litre
1X TAE buffer
- 20mls of 50X TAE
- Diluted with deionised water up to 1 litre

10X TBE

Separating Gel buffer
- 1.5M Tris pH 8.8

Stacking Gel buffer
- 1M Tris pH 6.8

5X PAGE buffer
30g Tris/l
144g glycine/l
100ml 10% SDS/l

SDS gel-loading dye
- 1mM Tris.Cl (pH 6.8)
- 200mM dithiothreitol
- 4% SDS (electrophoresis grade)
- 0.2% bromophenol blue
- 20% glycerol
**Luria Broth**

- 10g Tryptone
- 5g Yeast extract
- 10 g NaCl

- Mixed together in 1 litre of deionised water and autoclaved at 120°C for 20 minutes.

**LB-ampicillin agar plates**

- 10g Tryptone
- 5g Yeast extract
- 10g NaCl
- 15g Agar

- Mixed together in 1 litre of deionised water and autoclaved at 120°C for 20 minutes.

- 100mg/1L of ampicillin was added to the LB-agar which was cooled to approximately 50°C and the agar was poured gently onto plates aseptically.

**Transformation Buffer 1**

- 30mM KCH$_3$COO  \[2.97g/1000ml\]
- 10mM CaCl$_2$  \[1.47g/1000ml\]
- 50mM MnCl$_2$  \[9.90g/1000ml\]
- 100mM RbCl  \[12.09g/1000ml\]
- 15% Glycerol  \[150g/1000ml\]

- Adjust pH to 5.8 with 1M acetic acid, filter sterilse (0.2μM) and store at 4°C.
**Transformation buffer 2**

- 10mM MOPS  
  2.09g/1000ml
- 75mM CaCl₂  
  11.30g/1000ml
- 0mM RbCl  
  1.21g/1000ml
- 15% Glycerol  
  150g/1000ml

- Adjust pH to 6.5 with 1M KOH, filter sterilise (0.2μM) and store at 4°C.

**CELL AND TISSUE CULTURE REAGENTS**

**Phosphate buffered Saline**

- 1.95g Sodium dihydrogen orthophosphate dehydrate.
- 5.35g disodium hydrogen orthophosphate
- 42.5g Sodium Chloride

-Added to 5 litres of deionised water.

**10% Neutral buffered formalin**

- 4g Sodium dihydrogen phosphate monohydrate
- 6.5g Disodium hydrogen phosphate anhydrous
- 100ml 40% Formaldehyde

-Added to 900mls of deionised water
Glycergel
- 10g Gelatine
- 60ml Distilled water
- 70ml of Glycerol
- 0.25g of Phenol

Carbol Fuschin
- 10mls of carbol fuschin and 40mls of tap water
- The carbol fuschin dilution was filtered through filter paper before use.

ANTIBODIES

**BCL₂** 20μl in 1ml of PBS

**cMyc** 10μl in 1.5ml of PBS

**P53** 50μl in 950μl of PBS
# APPENDICES II

## Methods

### SDS-PAGE Gels

<table>
<thead>
<tr>
<th>Separating Gel</th>
<th>12% (mls)</th>
<th>10% (mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>4</td>
<td>3.2</td>
</tr>
<tr>
<td>1.5M Tris pH8.8</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>10% APES*</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED*</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking Gel</th>
<th>4% (mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
<td>3.6</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>0.65</td>
</tr>
<tr>
<td>1M Tris pH 6.8</td>
<td>0.63</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% APES*</td>
<td>0.075</td>
</tr>
<tr>
<td>TEMED*</td>
<td>0.007</td>
</tr>
</tbody>
</table>

* Added last few seconds as they are agents for setting the gels
Preparation of LB-ampicillin plates for transformation with pGem-T easy vector.

- The LB-ampicillin plates were dried at 37°C for 1-2 hours.
- Isopropyl-beta-D-galactose (IPTG) was made up to a concentration of 100mM diluted with deionised water. 100μl of IPTG was aseptically pipetted and spread onto a dry LB-ampicillin plate.
- X-galactose (X-Gal) was made up to a concentration of 20mg/ml diluted with dimethylsulfoxide (DMSO). 50μl of X-Gal was aseptically pipetted and spread onto a dry LB-ampicillin plate.

Preparation of competent cells (DH5α)

1. A single colony of cells was inoculated from an LB plate in 2.5mls of LB medium and incubated overnight at 37°C with shaking (approximately 225rpm)
2. The entire overnight culture was inoculated in 100mls of LB medium containing 20mM MgSO₄ for a 1:100 dilution. The cells were grown in a 1L flask until the optical density (A600) reached 0.4-0.6. (Typically 5-6 hours).
3. The cells were pelleted by centrifugation at 4,500 x g for 5 minutes at 4°C.
4. The cells were gently resuspended in 0.4 volume (based on the original culture volume) of ice-cold TFB 1. (All procedures done on ice for cold conditions)
5. The cells were pelleted by centrifugation at 4,500 x g for 5 minutes at 4°C.
6. The cells were gently resuspended in 1/25 of the original culture volume of ice-cold TFB 2.
The cells were incubated on ice for 15-60 minutes and aliquoted in 100μl per tube and quick frozen in the tubes in a dry-ice bath. The cells were stored at -70°C until required.

**Preparation of glycerol stocks from bacterial cultures**

1. 300μl of sterile glycerol was pipetted into sterile Eppendorf tubes (Eppendorf GmbH, Hamburg, Germany).
2. 700μl of bacterial cell culture was added to the Eppendorf tubes containing the glycerol (BDH) and was mixed by vortexing.
3. The tube was immediately placed on dry ice for quick freezing.
4. The glycerol bacterial cultures stocks were stored at -70°C.

**Preparation of mammalian cells for storage (aseptic procedure)**

1. Adherent cells were removed from the base of the flask by trypsination (described in chapter 3) and were resuspended in 10mls of cell culture medium.
2. All cells in suspension (trypsinised adherent cells and lymphoblastoid cell lines) were pelleted by centrifugation at 1200rpm for 10 minutes at 4°C.
3. 90μl of DMSO (Sigma-Aldrich) was added to pre-labelled cryovials (Falcon).
4. The pelleted cells were resuspended in 910μl of foetal calf serum (Gibco Invitrogen corporation) and added to the cryovials containing the DMSO. The tube was mixed gently and frozen at -70°C for 4-5 hours.
5. The cells were then placed in liquid nitrogen for long term storage.
Resusitation of cell lines from liquid nitrogen

1. The cryovial containing the cells was carefully taken out of liquid nitrogen and placed on the bench at room temperature for 1 minute.

2. The cryovial was then placed into warm water to defrost.

3. The 1ml volume of cells was pipetted from the cryovial into approximately 200mls of prewarmed (37°C) cell culture media (appropriate for the particular cells).
### APPENDICES III

*All appendices III in order of chapters*

**Table A.1** Intra-variation control raw data constituting the G2 scores

<table>
<thead>
<tr>
<th>Sample (code)</th>
<th>Non-IR Control 1</th>
<th>Non-IR Control 2</th>
<th>IR Control 1</th>
<th>IR Control 2</th>
<th>G2 Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN-1</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>34</td>
<td>90</td>
</tr>
<tr>
<td>CN-2</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>42</td>
<td>92</td>
</tr>
<tr>
<td>CN-3</td>
<td>0</td>
<td>0</td>
<td>38</td>
<td>52</td>
<td>90</td>
</tr>
<tr>
<td>CN-4</td>
<td>0</td>
<td>0</td>
<td>52</td>
<td>50</td>
<td>102</td>
</tr>
<tr>
<td>CN-5</td>
<td>0</td>
<td>2</td>
<td>34</td>
<td>68</td>
<td>100</td>
</tr>
<tr>
<td>CN-6</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>CN-6</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>CN-8</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>50</td>
<td>98</td>
</tr>
<tr>
<td>CN-9</td>
<td>0</td>
<td>0</td>
<td>44</td>
<td>54</td>
<td>98</td>
</tr>
<tr>
<td>CN-10</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>52</td>
<td>102</td>
</tr>
</tbody>
</table>

**Note:** All values are the number of aberrations per 50 metaphases except the final G2 scores (in bold). This was the sum of the irradiated (IR) controls minus the sum of the non-irradiated controls per sample.
Table A.2 Inter-variation control raw data constituting the G2 scores

<table>
<thead>
<tr>
<th>Sample (Code)</th>
<th>Non-IR Control 1</th>
<th>Non-IR Control 2</th>
<th>IR Control 1</th>
<th>IR Control 2</th>
<th>G2 Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-1</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>34</td>
<td>66</td>
</tr>
<tr>
<td>CI-2</td>
<td>0</td>
<td>0</td>
<td>26</td>
<td>44</td>
<td>70</td>
</tr>
<tr>
<td>CI-3</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>52</td>
<td>84</td>
</tr>
<tr>
<td>CI-4</td>
<td>2</td>
<td>0</td>
<td>38</td>
<td>48</td>
<td>84</td>
</tr>
<tr>
<td>CI-5</td>
<td>0</td>
<td>2</td>
<td>52</td>
<td>52</td>
<td>102</td>
</tr>
<tr>
<td>CI-6</td>
<td>0</td>
<td>2</td>
<td>26</td>
<td>52</td>
<td>76</td>
</tr>
<tr>
<td>CI-7</td>
<td>0</td>
<td>4</td>
<td>52</td>
<td>54</td>
<td>102</td>
</tr>
<tr>
<td>CI-8</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>50</td>
<td>110</td>
</tr>
<tr>
<td>CI-9</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>38</td>
<td>80</td>
</tr>
<tr>
<td>CI-10</td>
<td>0</td>
<td>0</td>
<td>36</td>
<td>32</td>
<td>68</td>
</tr>
</tbody>
</table>

Note: All values are the number of aberrations per 50 metaphases except the final G2 scores (in bold). This was the sum of the irradiated (IR) controls minus the sum of the non-irradiated controls per sample.

Table A.3 Statistical comparison of G2 scores in controls

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Intra-variation G2 scores</th>
<th>Inter-variation G2 scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>97.2</td>
<td>84.2</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>4.7</td>
<td>15.5</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>4.80%</td>
<td>18.24%</td>
</tr>
</tbody>
</table>

235
<table>
<thead>
<tr>
<th>Patient (code)</th>
<th>Non-IR Sample 1</th>
<th>Non-IR Sample 2</th>
<th>IR Sample 1</th>
<th>IR Sample 2</th>
<th>G2 Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1</td>
<td>0</td>
<td>0</td>
<td>110</td>
<td>82</td>
<td>192*</td>
</tr>
<tr>
<td>BC-2</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>BC-3</td>
<td>0</td>
<td>2</td>
<td>80</td>
<td>82</td>
<td>160*</td>
</tr>
<tr>
<td>BC-4</td>
<td>0</td>
<td>2</td>
<td>130</td>
<td>104</td>
<td>242*</td>
</tr>
<tr>
<td>BC-5</td>
<td>0</td>
<td>0</td>
<td>94</td>
<td>80</td>
<td>174*</td>
</tr>
<tr>
<td>BC-6</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>56</td>
<td>126</td>
</tr>
<tr>
<td>BC-7</td>
<td>0</td>
<td>0</td>
<td>94</td>
<td>102</td>
<td>196*</td>
</tr>
<tr>
<td>BC-8</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>50</td>
<td>106</td>
</tr>
<tr>
<td>BC-9</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>BC-10</td>
<td>0</td>
<td>0</td>
<td>54</td>
<td>58</td>
<td>112</td>
</tr>
<tr>
<td>BC-11</td>
<td>0</td>
<td>0</td>
<td>88</td>
<td>80</td>
<td>168*</td>
</tr>
<tr>
<td>BC-12</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>90</td>
<td>146*</td>
</tr>
<tr>
<td>BC-13</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>100</td>
<td>130</td>
</tr>
<tr>
<td>BC-14</td>
<td>0</td>
<td>0</td>
<td>68</td>
<td>56</td>
<td>124</td>
</tr>
<tr>
<td>BC-15</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>88</td>
<td>170*</td>
</tr>
<tr>
<td>BC-16</td>
<td>0</td>
<td>0</td>
<td>78</td>
<td>82</td>
<td>160*</td>
</tr>
<tr>
<td>BC-17</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>58</td>
<td>120</td>
</tr>
<tr>
<td>BC-18</td>
<td>0</td>
<td>2</td>
<td>46</td>
<td>74</td>
<td>118</td>
</tr>
<tr>
<td>BC-19</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>70</td>
<td>132</td>
</tr>
<tr>
<td>BC-20</td>
<td>0</td>
<td>0</td>
<td>72</td>
<td>90</td>
<td>162*</td>
</tr>
<tr>
<td>BC-21</td>
<td>0</td>
<td>2</td>
<td>56</td>
<td>50</td>
<td>104</td>
</tr>
<tr>
<td>BC-22</td>
<td>0</td>
<td>0</td>
<td>102</td>
<td>84</td>
<td>186*</td>
</tr>
<tr>
<td>BC-23</td>
<td>0</td>
<td>0</td>
<td>82</td>
<td>70</td>
<td>152*</td>
</tr>
<tr>
<td>BC-24</td>
<td>0</td>
<td>0</td>
<td>58</td>
<td>52</td>
<td>110</td>
</tr>
<tr>
<td>BC-25</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>BC-26</td>
<td>0</td>
<td>0</td>
<td>36</td>
<td>56</td>
<td>92</td>
</tr>
<tr>
<td>BC-27</td>
<td>0</td>
<td>0</td>
<td>82</td>
<td>88</td>
<td>170*</td>
</tr>
</tbody>
</table>
Note: All values are the number of aberrations per 50 metaphases except the final G2 scores (in bold). This was the sum of the irradiated (IR) controls minus the sum of the non-irradiated controls per sample. * denotes the G2 scores that exceeded the G2 radiosensitivity cut-off value (130 aberrations/100 metaphases) and were recorded as G2 radiosensitive.

Table A.5 Prostate cancer and benign prostatic hyperplasia raw G2 scores

<table>
<thead>
<tr>
<th>Prostate Cancer G2 Scores</th>
<th>Benign Prostatic hyperplasia G2 Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Code</td>
<td>G2 score</td>
</tr>
<tr>
<td>DPC-1</td>
<td>142</td>
</tr>
<tr>
<td>DPC-2</td>
<td>212</td>
</tr>
<tr>
<td>DPC-3</td>
<td>116</td>
</tr>
<tr>
<td>DPC-4</td>
<td>186</td>
</tr>
<tr>
<td>DPC-5</td>
<td>194</td>
</tr>
<tr>
<td>DPC-6</td>
<td>172</td>
</tr>
<tr>
<td>QPC-1</td>
<td>156</td>
</tr>
<tr>
<td>QPC-2</td>
<td>190</td>
</tr>
<tr>
<td>QPC-3</td>
<td>136</td>
</tr>
<tr>
<td>QPC-4</td>
<td>126</td>
</tr>
<tr>
<td>QPC-5</td>
<td>182</td>
</tr>
<tr>
<td>QPC-6</td>
<td>160</td>
</tr>
<tr>
<td>QPC-7</td>
<td>108</td>
</tr>
<tr>
<td>QPC-8</td>
<td>170</td>
</tr>
<tr>
<td>QPC-9</td>
<td>116</td>
</tr>
<tr>
<td>QPC-10</td>
<td>120</td>
</tr>
<tr>
<td>QPC-11</td>
<td>171</td>
</tr>
<tr>
<td>QPC-12</td>
<td>146</td>
</tr>
</tbody>
</table>
Table A.6  % Colony formation ± standard error of cell survival of prostate adherent cell lines (PC3 and DU145) in contrast to control cell lines (HPV-G) exposed to ionising radiation.

<table>
<thead>
<tr>
<th>Adherent cell lines</th>
<th>0Gy</th>
<th>0.5Gy</th>
<th>1gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control – HPV-G</td>
<td>100 ± 6.78</td>
<td>68.29 ± 1.16</td>
<td>64.43 ± 6.37</td>
</tr>
<tr>
<td>PC3</td>
<td>100 ± 10.03</td>
<td>38.33 ± 2.30</td>
<td>53.02 ± 8.81</td>
</tr>
<tr>
<td>DU145</td>
<td>100 ± 12.62</td>
<td>87.04 ± 4.17</td>
<td>66.54 ± 5.20</td>
</tr>
</tbody>
</table>

Note: All values represent the mean ± SE for n=3. All values expressed as a percentage of control and control is expressed as 100%.

Table A.7  % Survival ± standard error of prostate lymphoblastoid cell lines (QPC-2, QPC-5 and QPC-9) in contrast to control lymphoblastoid cell lines (C2ABR) exposed to ionising radiation.

<table>
<thead>
<tr>
<th>Prostate lymphoblastoid cell lines</th>
<th>0Gy</th>
<th>0.5Gy</th>
<th>1Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2ABR</td>
<td>100 ± 0.63</td>
<td>71.69 ± 1.01</td>
<td>58.65 ± 1.20</td>
</tr>
<tr>
<td>QPC-2</td>
<td>100 ± 1.12</td>
<td>41.97 ± 2.46</td>
<td>31.86 ± 1.46</td>
</tr>
<tr>
<td>QPC-5</td>
<td>100 ± 3.19</td>
<td>44.95 ± 5.89</td>
<td>34.39 ± 1.86</td>
</tr>
<tr>
<td>QPC-9</td>
<td>100 ± 1.44</td>
<td>70.93 ± 7.12</td>
<td>48.85 ± 5.87</td>
</tr>
</tbody>
</table>

Note: All values represent the mean ± SE for n=3. All values expressed as a percentage of control and control is expressed as 100%.
Table A.6 Spectrophotometry data and preparation of breast cancer DNA samples for dHPLC mutational analysis

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>260nm value</th>
<th>Conc in ng/µl</th>
<th>µl of DNA for 500ng</th>
<th>µl of Tris (pH 8) to 25µl vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.1442</td>
<td>721</td>
<td>0.69</td>
<td>24.31</td>
</tr>
<tr>
<td>A2</td>
<td>0.0817</td>
<td>409</td>
<td>1.22</td>
<td>23.78</td>
</tr>
<tr>
<td>A3</td>
<td>0.2387</td>
<td>119</td>
<td>4.19</td>
<td>20.81</td>
</tr>
<tr>
<td>A4</td>
<td>0.1135</td>
<td>568</td>
<td>0.88</td>
<td>24.12</td>
</tr>
<tr>
<td>A5</td>
<td>0.0686</td>
<td>343</td>
<td>1.46</td>
<td>23.54</td>
</tr>
<tr>
<td>A6</td>
<td>0.0765</td>
<td>383</td>
<td>1.31</td>
<td>23.69</td>
</tr>
<tr>
<td>A7</td>
<td>0.1045</td>
<td>523</td>
<td>0.96</td>
<td>24.04</td>
</tr>
<tr>
<td>A8</td>
<td>0.2262</td>
<td>1131</td>
<td>0.44</td>
<td>24.56</td>
</tr>
<tr>
<td>B1</td>
<td>0.0652</td>
<td>326</td>
<td>1.53</td>
<td>23.47</td>
</tr>
<tr>
<td>B2</td>
<td>0.0876</td>
<td>438</td>
<td>1.14</td>
<td>23.86</td>
</tr>
<tr>
<td>B3</td>
<td>0.1321</td>
<td>661</td>
<td>0.76</td>
<td>24.24</td>
</tr>
<tr>
<td>B4</td>
<td>0.0686</td>
<td>343</td>
<td>1.46</td>
<td>23.54</td>
</tr>
<tr>
<td>B5</td>
<td>0.1102</td>
<td>551</td>
<td>0.91</td>
<td>24.09</td>
</tr>
<tr>
<td>B6</td>
<td>0.1169</td>
<td>585</td>
<td>0.86</td>
<td>24.14</td>
</tr>
<tr>
<td>B7</td>
<td>0.0536</td>
<td>268</td>
<td>1.87</td>
<td>23.13</td>
</tr>
<tr>
<td>C1</td>
<td>0.1041</td>
<td>521</td>
<td>0.96</td>
<td>24.04</td>
</tr>
<tr>
<td>C2</td>
<td>0.0807</td>
<td>404</td>
<td>1.24</td>
<td>23.76</td>
</tr>
<tr>
<td>C3</td>
<td>0.0741</td>
<td>371</td>
<td>1.35</td>
<td>23.65</td>
</tr>
<tr>
<td>C4</td>
<td>0.1089</td>
<td>545</td>
<td>0.92</td>
<td>24.08</td>
</tr>
<tr>
<td>C5</td>
<td>0.1264</td>
<td>632</td>
<td>0.79</td>
<td>24.21</td>
</tr>
<tr>
<td>C6</td>
<td>0.1010</td>
<td>505</td>
<td>0.99</td>
<td>24.01</td>
</tr>
<tr>
<td>C7</td>
<td>0.2208</td>
<td>1104</td>
<td>0.45</td>
<td>24.55</td>
</tr>
<tr>
<td>D1</td>
<td>0.1264</td>
<td>632</td>
<td>0.79</td>
<td>24.21</td>
</tr>
<tr>
<td>Sample Code</td>
<td>260nm value</td>
<td>Conc in ng/μl</td>
<td>μl of DNA for 500ng</td>
<td>μl of Tris (pH 8) to 25μl vol.</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>---------------</td>
<td>---------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>D2</td>
<td>0.1071</td>
<td>536</td>
<td>0.93</td>
<td>24.07</td>
</tr>
<tr>
<td>D3</td>
<td>0.0646</td>
<td>323</td>
<td>1.55</td>
<td>23.45</td>
</tr>
<tr>
<td>D4</td>
<td>0.2011</td>
<td>1006</td>
<td>0.50</td>
<td>24.50</td>
</tr>
<tr>
<td>D5</td>
<td>0.1264</td>
<td>632</td>
<td>0.79</td>
<td>24.21</td>
</tr>
<tr>
<td>D6</td>
<td>0.1010</td>
<td>505</td>
<td>0.99</td>
<td>24.01</td>
</tr>
<tr>
<td>D7</td>
<td>0.2208</td>
<td>1104</td>
<td>0.45</td>
<td>24.55</td>
</tr>
<tr>
<td>E1</td>
<td>0.0374</td>
<td>187</td>
<td>2.67</td>
<td>22.33</td>
</tr>
<tr>
<td>E2</td>
<td>0.1689</td>
<td>845</td>
<td>0.59</td>
<td>24.41</td>
</tr>
<tr>
<td>E3</td>
<td>0.1327</td>
<td>664</td>
<td>0.75</td>
<td>24.25</td>
</tr>
<tr>
<td>E4</td>
<td>0.2736</td>
<td>1368</td>
<td>0.37</td>
<td>24.63</td>
</tr>
<tr>
<td>E5</td>
<td>0.1967</td>
<td>984</td>
<td>0.51</td>
<td>24.49</td>
</tr>
<tr>
<td>E6</td>
<td>0.0436</td>
<td>218</td>
<td>2.29</td>
<td>22.71</td>
</tr>
<tr>
<td>E7</td>
<td>0.1116</td>
<td>558</td>
<td>0.90</td>
<td>24.10</td>
</tr>
<tr>
<td>F1</td>
<td>0.0230</td>
<td>115</td>
<td>4.35</td>
<td>20.65</td>
</tr>
<tr>
<td>F2</td>
<td>0.0529</td>
<td>265</td>
<td>1.89</td>
<td>23.11</td>
</tr>
<tr>
<td>F3</td>
<td>0.2097</td>
<td>1049</td>
<td>0.48</td>
<td>24.52</td>
</tr>
<tr>
<td>F4</td>
<td>0.1308</td>
<td>654</td>
<td>0.76</td>
<td>24.24</td>
</tr>
<tr>
<td>F5</td>
<td>0.1160</td>
<td>580</td>
<td>0.86</td>
<td>24.24</td>
</tr>
<tr>
<td>F6</td>
<td>0.0904</td>
<td>452</td>
<td>1.11</td>
<td>23.89</td>
</tr>
<tr>
<td>F7</td>
<td>0.1860</td>
<td>930</td>
<td>0.54</td>
<td>24.46</td>
</tr>
<tr>
<td>G1</td>
<td>0.1679</td>
<td>840</td>
<td>0.60</td>
<td>24.40</td>
</tr>
<tr>
<td>G2</td>
<td>0.1595</td>
<td>798</td>
<td>0.63</td>
<td>24.37</td>
</tr>
<tr>
<td>G3</td>
<td>0.0973</td>
<td>487</td>
<td>1.03</td>
<td>23.97</td>
</tr>
<tr>
<td>G4</td>
<td>0.0948</td>
<td>474</td>
<td>1.05</td>
<td>23.95</td>
</tr>
<tr>
<td>G5</td>
<td>0.0745</td>
<td>373</td>
<td>1.34</td>
<td>23.66</td>
</tr>
<tr>
<td>G6</td>
<td>0.1446</td>
<td>723</td>
<td>0.69</td>
<td>24.31</td>
</tr>
<tr>
<td>Sample Code</td>
<td>260nm value</td>
<td>Conc in ng/μl</td>
<td>μl of DNA for 500ng</td>
<td>μl of Tris (pH 8) to 25μl vol.</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>---------------</td>
<td>---------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>G7</td>
<td>0.1676</td>
<td>838</td>
<td>0.60</td>
<td>24.40</td>
</tr>
<tr>
<td>H1</td>
<td>0.1511</td>
<td>756</td>
<td>0.66</td>
<td>24.34</td>
</tr>
<tr>
<td>H2</td>
<td>0.0611</td>
<td>306</td>
<td>1.64</td>
<td>23.36</td>
</tr>
<tr>
<td>H3</td>
<td>0.0635</td>
<td>318</td>
<td>1.57</td>
<td>23.43</td>
</tr>
<tr>
<td>H4</td>
<td>0.0677</td>
<td>339</td>
<td>1.48</td>
<td>23.52</td>
</tr>
<tr>
<td>H5</td>
<td>0.0865</td>
<td>433</td>
<td>1.16</td>
<td>23.84</td>
</tr>
<tr>
<td>H6</td>
<td>0.1340</td>
<td>670</td>
<td>0.75</td>
<td>24.25</td>
</tr>
<tr>
<td>H7</td>
<td>0.1029</td>
<td>515</td>
<td>0.97</td>
<td>24.03</td>
</tr>
</tbody>
</table>

Table A.7 Raw data of cell survival of ATL2ABR cells in contrast to A-T cells (AT25ABR and AT25ABR) and control (C2ABR) exposed to ionising radiation.

<table>
<thead>
<tr>
<th>Lymphoblastoid cell line</th>
<th>0Gy</th>
<th>0.5Gy</th>
<th>1Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2ABR</td>
<td>100 ± 1.35</td>
<td>88.64 ± 6.14</td>
<td>62.47 ± 8.27</td>
</tr>
<tr>
<td>AT25ABR</td>
<td>100 ± 1.76</td>
<td>46.65 ± 0.94</td>
<td>31.62 ± 4.46</td>
</tr>
<tr>
<td>AT1ABR</td>
<td>100 ± 1.05</td>
<td>47.98 ± 7.91</td>
<td>37.83 ± 5.99</td>
</tr>
<tr>
<td>ATL2ABR</td>
<td>100 ± 6.84</td>
<td>72.30 ± 6.43</td>
<td>47.98 ± 2.93</td>
</tr>
</tbody>
</table>

Note: All values represent the mean ± SE for n=3. All values expressed as a percentage of control and control is expressed as 100%.
Table A.8 Raw data of cell survival of ATL2ABR cells transfected with the short form of \textit{APTX} in contrast to A-T cells (AT25ABR) and control cells (C2ABR) exposed to ionising radiation.

<table>
<thead>
<tr>
<th>Lymphoblastoid cell line</th>
<th>0 Gy</th>
<th>0.5 Gy</th>
<th>1 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2ABR</td>
<td>100 ± 3.87</td>
<td>80.98 ± 1.84</td>
<td>66.87 ± 3.60</td>
</tr>
<tr>
<td>AT25ABR</td>
<td>100 ± 4.58</td>
<td>57.68 ± 2.51</td>
<td>33.54 ± 8.20</td>
</tr>
<tr>
<td>ATL2ABR-Trans</td>
<td>100 ± 2.73</td>
<td>70.72 ± 3.90</td>
<td>55.12 ± 10.64</td>
</tr>
</tbody>
</table>

\textbf{Note:} All values represent the mean ± SE for \( n = 3 \). All values expressed as a percentage of control and control is expressed as 100%.

Table A.9 Comparison of % survival ± standard error of ATL2ABR cells (untransfected and transfected) exposed to ionising radiation

<table>
<thead>
<tr>
<th>ATL2ABR cells</th>
<th>0 Gy</th>
<th>0.5 Gy</th>
<th>1 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Colony formation ± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untransfected ATL2ABR cells</td>
<td>100 ± 6.84</td>
<td>72.30 ± 6.43</td>
<td>47.98 ± 2.93</td>
</tr>
<tr>
<td>Transfected ATL2ABR cells</td>
<td>100 ± 2.73</td>
<td>70.72 ± 3.90</td>
<td>55.12 ± 10.64</td>
</tr>
</tbody>
</table>

\textbf{Note:} All values represent the mean ± SE for \( n = 3 \). All values expressed as a percentage of control and control is expressed as 100%.
Table A.10  % Survival ± standard error of L939 cells (AOA1) transfected with the short form of APTX (with and without CdCl₂ induction) and mutated (V263G) short form of APTX and compared to control cells (C2ABR); all exposed to hydrogen peroxide.

<table>
<thead>
<tr>
<th>Lymphoblastoid cell lines</th>
<th>0mM</th>
<th>0.5mM</th>
<th>1mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L939-S\textit{APTX} (- CdCl₂)</td>
<td>100 ± 3.36</td>
<td>34.60 ± 0.73</td>
<td>30.77 ± 1.43</td>
</tr>
<tr>
<td>L939-S\textit{APTX} (+ CdCl₂)</td>
<td>100 ± 8.75</td>
<td>53.72 ± 5.19</td>
<td>46.58 ± 1.64</td>
</tr>
<tr>
<td>L939-V263G in S\textit{APTX} (+CdCl₂)</td>
<td>100 ± 2.13</td>
<td>33.40 ± 3.19</td>
<td>29.26 ± 1.15</td>
</tr>
<tr>
<td>C2ABR</td>
<td>100 ± 1.18</td>
<td>57.96 ± 1.93</td>
<td>52.43 ± 1.61</td>
</tr>
<tr>
<td>C2ABR-S\textit{APTX} (+CdCl₂)</td>
<td>100 ± 1.17</td>
<td>60.33 ± 1.98</td>
<td>52.88 ± 3.22</td>
</tr>
</tbody>
</table>

\textbf{Note:} All values represent the mean ± SE for n=3. All values expressed as a percentage of control and control is expressed as 100%.

Table A.11  % Survival ± standard error of L939 cells (AOA1) transfected with EGFP constructs containing long form of APTX and mutated (V263G) long form of APTX in contrast to control cells (C2ABR); all exposed to hydrogen peroxide.

<table>
<thead>
<tr>
<th>Lymphoblastoid cell line</th>
<th>0mM</th>
<th>0.5mM</th>
<th>1mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2ABR</td>
<td>100 ± 1.69</td>
<td>49.10 ± 1.71</td>
<td>33.56 ± 2.15</td>
</tr>
<tr>
<td>L939-EGFP</td>
<td>100 ± 0.41</td>
<td>34.92 ± 0.84</td>
<td>27.06 ± 0.72</td>
</tr>
<tr>
<td>L939-EGFP with L\textit{APTX}</td>
<td>100 ± 3.90</td>
<td>46.80 ± 1.33</td>
<td>28.87 ± 1.28</td>
</tr>
<tr>
<td>L939-EGFP with V263G in L\textit{APTX}</td>
<td>100 ± 1.70</td>
<td>33.21 ± 1.54</td>
<td>28.97 ± 1.23</td>
</tr>
</tbody>
</table>

\textbf{Note:} All values represent the mean ± SE for n=3. All values expressed as a percentage of control and control is expressed as 100%.
REFERENCES


• ATCC website: http://www.lgepromochem.com/atcc/


• DiDonato S, Gellerra C and Mariotti C, 2001. The complex clinical and
genetic classification of inherited ataxias II. Autosomal recessive ataxias.
Neurological science, 22: 219-228.

Correlation between cellular radiosensitivity and non-repaired double-strand
breaks studied in nine mammalian cell lines. International journal of radiation
biology. 73(3): 269-278.

• Dipple A, 1995. DNA adducts of chemical carcinogens. Carcinogenesis. 16:
437-441.

• Dizdaroglu M, 1999. Mechanisms of oxidative damage: Lesions and their
measurement in: Dizdaroglu M, Karakaya A, editors. Advances in DNA
damage and Repair, kluwer Academic publishers/Plenum press, New York.
67-87.

Qian C, Marks A.F, Slager S.L, Peterson B.J, Smith D.I, Cheville J.C, Blute
Mutations in CHEK 2 associated with prostate cancer risk. American journal
of human genetics, 72: 270-280.


• Hall E.J, 2000. Radiobiology for the radiologist, fifth edition, Lippincott Williams and Wilkins, NY, USA.


• Irish Cancer Society website:


H, Birnbaum D, 1995. Loss of heterozygosity and linkage analysis in breast
carcinoma: indication for a putative third susceptibility gene on the short arm
of chromosome 8. Oncogene, 10: 1023-1026.

with wide-ranging implications in tissue kinetics. British Journal of Cancer,
26: 239-257.

• Kim S.T, Xu B, Kastan M.B, 2002. Involvement of the cohesion protein,
Smc1, in Atm-dependent and independent responses to DNA damage. Genes
and development, 16(5): 560-570.

Reconstitution of DNA base excision-repair with purified human proteins:
interaction between DNA polymerase beta and the XRCC1 protein. The
EMBO Journal, 15: 6662-6670.

• Lane D.P, 1994. p53 and human cancers. British Medical Bull. 50(3): 582-
99.


• Marples B, Longhurst D, Eastham A.M, West C.M, 1998. The ratio of initial/residual DNA damage predicts intrinsic radiosensitivity of seven cervix carcinoma cell lines. British journal of cancer, **77(7)**: 1108-1114.


haplotype analysis: \((\text{CCG})_{1,2}\) polymorphism and contribution to founder effect. Journal of medical genetics, 36: 112-114.


dehinctic modulation of human prostate epithelial cells by growth inhibitory

• Peehl D.M, Stamey T.A, 1986. Serum-free growth of adult human prostate

Cdc25C on serine 216 and promotes 14-3-3 protein binding. Cell growth
differentiation, 9: 197-208.

• Pharoah P.D, 2003. Genetic susceptibility, predicting risk and preventing
cancer. Recent results in cancer research, 163: 7-18; discussion 264-6.

• Picksley S.M, Lane D.P, 1993. The p53-mdm2 autoregulatory feedback loop:
a paradigm for the regulation of growth control by p53? Bioessays. 15(10):
689-90.


• Pines J, 1996. Cyclins from sea urchins to HeLas: Making the human cell


- Solveig Walles S.A and Erixon K, 1984. Single-strand breaks in DNA of various organs of mice induced by methyl methanesulfonate and
dimethylsulfoxide determined by the alkaline unwinding technique.
Carcinogenesis, 5: 319-323.


to the human ATM 7636del9 common mutation exhibit a variant phenotype. Cancer research, 61: 4561-4568.


