A study of the role of *ATM* mutations in the pathogenesis of B-cell chronic lymphocytic leukaemia

by

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Synopsis

Mutations in the *ATM* gene have previously been identified in CLL tumours. In this project, I have demonstrated that their detection would have prognostic value. With a prevalence of 12%, *ATM* mutations represent the commonest single gene defect to be detected in CLL tumours and they identified a subgroup of CLL patients that had a significant reduction in both treatment free and overall survival. Furthermore, *ATM* mutations provided prognostic information that was independent of age, clinical stage, the mutation status of the *IGVH* genes and *TP53* mutations.

The temporal acquisition of the *ATM* mutations and their relationship with loss of an *ATM* allele via a chromosomal 11q deletion provides clues into their mechanism of action. There was only a partial correlation between CLL tumours with mutations in the *ATM* gene and those with a chromosome 11q deletion. In certain cases, the *ATM* mutations represented germ-line changes and in others were acquired very early in the disease course raising the possibility that they might contribute to the initial clonal transformation process. However, in some CLL tumours, the *ATM* mutations had been acquired after the development of the tumour clone during disease progression indicating that there may be a step-wise acquisition of *ATM* allelic defects during the ontogeny of CLL.

The ATM protein is the key coordinator of the cellular response to DNA double strand breaks. In this study, I showed that bi-allelic defects in the *ATM* gene lead to deficient ATM dependent responses, including the up regulation of p53, following both ionising irradiation and also treatment with the

chemotherapeutic drug, Fludarabine. Thus an important mechanism accounting for the poor outcome in CLL patients with *ATM* mutations is likely to relate to chemo-resistance. Interestingly, there were differential responses to DNA damage with both irradiation and fludarabine amongst the category of tumours with an 11q deletion according to the status of the remaining *ATM* allele. Therefore, *ATM* mutations can stratify tumours with a chromosome 11q deletion into two functional subgroups.

The identification of CLL tumours with *ATM* mutations would therefore predict those patients that will have a poor clinical outcome and be both more likely to require early treatment for their disease. Patients whose tumours had bi-allelic *ATM* defects will be expected to have deficient responses to DNA damaging chemotherapeutic drugs, while those with mono-allelic *ATM* defects might identify a group in whom the use of DNA damaging agents could provide selective pressure for the emergence of sub-clones that have subsequently acquired bi-allelic *ATM* defects.

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List of Abbreviations

ºC – Degree centigrade

A - Adenosine

a - ampere

ALL – Acute lymphoblastic leukaemia

Apaf – Apoptosis activating factor

AT - Ataxia Telangiectasia

ATM - Ataxia Telangiectasia mutated

ATP – Adenosine triphosphate

ATR - Ataxia Telangiectasia related protein

BCR – B cell receptor

BLM – Bloom

bp – base pair

BRCA1 – Breast Cancer associated gene 1

C - Cytosine

Caspase - Cysteine protease

CD – Cluster of differentiation

Chk – Checkpoint kinase

CLL- Chronic lymphocytic leukaemia

DHPLC - Denaturing high performance liquid chromatography

DMSO - Dimethyl Sulphoxide

DNA – Deoxyribose nucleic acid

DNA-PKcs - DNA protein kinase catalytic sub-unit

dNTP – deoxynucleotide tri-phosphate

dsDNA – double strand DNA

DSB – double strand DNA breaks

DTT - Dithiothreitol

EDTA - Ethylene diamine tetra-acetic acid

FACS – Fluoresent activated cell sorting

FCS - Fetal calf serum

FISH – Fluorescent in situ hybridisation

G - guanine

g - gram

gDNA – genomic DNA

Gy - gray

HSP – Heat shock protein

ICL – Inter-strand cross link

IG - immunoglobulin

IGVH - immunoglobulin variable heavy genes

IR – ionizing irradiation

kb - kilobase

kD - kilodalton

l – litre

LDT – lymphocyte doubling time

M – molar

m - milli

µ - micro

mb - megabase

MCL – mantle cell lymphoma

MDM2 – mouse

MRN – Mre11, Nbs1 and Rad 50 complex

mTOR - mammalian target of rapamycin

n - nano

Nbs1- Nijmegen Breakage syndrome

NFKB - NFkappaB

PAGE - Polyacrylamide Gel Electrophoresis

PBS - Phospho-buffered saline

PCR – Polymerase chain reaction

PIKK - phospho-inositide 3-kinase-like kinases

RNA – ribonucleic acid

rpm – rotations per minute

OS – Overall survival

SDS - Sodium Dodecyl Sulphate

SNP – Single nucleotide polymorphism

ssDNA – single strand DNA

T - Thymine

TCR – T cell receptor

T-PLL – T cell prolymphocytic leukaemia

TFS – Treatment free survival

TRRAP – Transactivation-transformation domain associated protein

UV – Ultra-violet

v – volt

Zap 70 - Zeta-associated protein 70

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Introduction

1.1 B-Cell Chronic Lymphocytic Leukaemia

B-cell chronic lymphocytic leukaemia (CLL) is the commonest leukaemia in the western world with an incidence of approximately 3 per 100,000 per year. The median age of onset is 55-60 years and it is approximately 1.5 fold more common in men than in women. The disease results from the clonal expansion of mature B-lymphocytes and the diagnosis of B-CLL is confirmed by the demonstration of a population of clonal B cells which have a distinctive pattern of cell surface and cytoplasmic markers. CLL tumours cells show restriction of kappa or lambda immunoglobulin light chain expression and can be distinguished from other B-cell lymphoproliferative disorders on the basis of their strong positive expression of CD5 and CD23, weak positive expression of surface immunoglobulin and absence of CD10 or CD79b expression (Oscier D *et al*, 2004).

1.2 Clinical heterogeneity of B-cell CLL

A characteristic feature of CLL is the marked clinical heterogeneity. This is manifest by variations in disease burden and distribution at diagnosis, and by variability in the subsequent rate and pattern of disease progression, the sensitivity to chemotherapeutic agents and the development of infective or immune complications.

1.2.1 Disease presentation

Many patients are asymptomatic at presentation and the diagnosis of CLL is made incidentally from the finding of a peripheral blood lymphocytosis. Symptomatic patients will typically present with a history of fatigue and sweats, and in some cases recurrent or prolonged infections. Lymphadenopathy may be present at diagnosis and this is usually symmetrical, painless and commonly involves the cervical, axillary or inguinal regions. Enlargement of the spleen or liver is associated with advanced disease but may rarely be present at diagnosis. CLL patients can also present with symptoms related to anaemia or thrombocytopenia and these may reflect extensive bone marrow infiltration with the CLL tumour clone or alternatively may result from autoimmune haemolytic anaemia or thrombocytopenia, which are both associated with CLL (D'Arena G *et al*. 2003).

1.2.2 Clinical Staging

The extent of the disease can be assessed using clinical staging systems. The Binet and Rai systems are commonly used and both categorise patients according to the degree of lymph node or organ involvement and the presence of peripheral blood cytopenias (Binet JL *et al*. 1981, Rai KR *et al*, 1975). Five anatomical regions are described, namely cervical, axillary, inguinal lymph nodes as well as spleen and liver. In the Binet system patients are categorised as stage A if they have involvement of less than three lymphoid areas, stage B if there is nodal expansion of between three and five lymphoid areas and stage C if there is either an anaemia (haemoglobin <10g/dl) or thrombocytopenia (platelet count < $100x10^9$/l), regardless of the number of lymphoid areas involved. To designate a patient as stage C,

implying extensive marrow infiltration with CLL, requires the exclusion of an immune cause for any cytopenia (Binet JL et al; 1981). Approximately 70-80% of patients have stage A disease at presentation and many of these patients will be asymptomatic with an isolated peripheral blood lymphocytosis (Oscier D *et al*, 2004).

1.2.3 Disease Progression

The subsequent disease course after diagnosis is also highly variable, particularly in those patients that present with stage A disease. Some patients remain asymptomatic and have an entirely stable CLL clone. These individuals never require treatment for their disease. Other patients have an expansion of their clone and this may occur insidiously over many years or can occur much more rapidly. Tumour cells may accumulate predominantly in the peripheral blood or primarily within the lymph nodes or the bone marrow, and this pattern of disease distribution is highly variable between individuals (Chiorazzi N *et al*, 2005, Hamblin TJ, 2005).

The principal indication for treatment in CLL patients is the presence of symptoms (Oscier D *et al*, 2004). CLL is incurable with chemotherapy and there is no evidence to indicate that early treatment of patients with limited stage disease has any beneficial effect on outcome. Indeed there is some data to suggest that this could actually have a detrimental effect (CLL Trialists' Collaborative Group, 1999). Therefore, asymptomatic CLL patients are usually managed by a ‘watch and wait’ policy and as such, many stage A CLL patients will never require treatment. The majority of patients with stage B or

C disease will be symptomatic and will invariably receive treatment (Oscier D *et al*, 2004).

1.2.4 Treatment of CLL patients

There are a number of chemotherapeutic agents, which have been shown to have activity in CLL and comparisons between different agents either alone or in combination continues to be the subject of clinical trials (Robak T *et al*, 2002, Schmitt B *et al*, 2002). The initial therapy used to treat CLL patients in the UK is usually chlorambucil or fludarabine alone or the combination of fludarabine with cyclophosphamide (Oscier D *et al*, 2004). These drugs all induce tumour cell death through mechanisms that involve DNA damage (Begleiter A *et al*, 1996, Pettitt AR, 2003, Yamauchi T *et al*, 2001). Patients will typically be treated with up to six cycles of a given therapy and the degree of any response can then be assessed and classified as a complete response, a nodular partial response, a partial response or no response.

Although, the majority of patients will gain some degree of response to these first lines of treatment, their disease will inevitably progress and at some stage most patients will require re-treatment (Oscier D *et al*, 2004). Commonly CLL tumour cells become less sensitive to these chemotherapeutic agents over time and alternative drugs and combinations will be required (Montserrat E, 2006). These will often include agents such as high dose methylprednisolone and more recently the monoclonal antibodies Rituximab (anti-CD20) and Alemtuzumab (anti-CD52), which have been shown to have efficacy in CLL and induce tumour cell apoptosis by alternative mechanisms

from the DNA damaging chemotherapeutic agents (Thornton PD *et al*, 2003, Byrd JC *et al*, 2002, Lozanski G *et al*, 2004). Eventually patients may fail to respond to any of the currently available agents and will die from progressive disease.

1.3 Clinical and Biological prognostic markers in CLL

Because of this marked clinical heterogeneity between different CLL patients, there has been considerable interest in identifying both the clinical and biological features of the disease that can help distinguish those patients that are likely to have an indolent, stable disease course from those that will have a progressive course. These markers are useful in the clinical setting for the counselling of patients at the time of diagnosis.

Importantly, however, the identification of prognostic features has also led to an increased understanding of the nature of CLL, both in terms of the cellular origins of the disease and the factors that influence the behaviour of the tumour clone (D'Arena G *et al*, 2003). In addition, by understanding the mechanisms of action of these biological prognostic markers it is likely that in the future they will increasingly be used to influence clinical decisions. This might lead to a situation where the choice of treatment for individual patients is made on the basis of the knowledge of specific molecular defects in their tumour cells (Binet JL *et al*, 2006). Finally, the understanding of the critical signalling pathways in CLL tumours that have been identified through these markers is already leading to the development of novel targeted agents (Kojima K *et al*, 2006, Alvi *et al*, 2005).

1.3.1 Clinical Features

Clinical features associated with an impaired outcome in CLL patients include older age, male sex, a short lymphocyte doubling time (LDT), advanced disease stage and a family history of CLL (Table 1.1). These clinical markers have predictive value and have also provided important insights into the nature of the disease.

1.3.1.1 Age and Gender

Age and gender are both predictors of outcome in CLL. The poor outcome associated with increasing age partly reflects co-morbidity rather than characteristics of the tumour cell population. By comparison, the phenotype of CLL tumours is clearly affected by gender, and CLL is both commoner in males than in females and progresses more rapidly, resulting in a greater likelihood of patients requiring treatment. The mechanisms that underlie the poorer outcome in males are not known (Catovsky D *et al*, 1989, Molica S *et al*, 2005).

1.3.1.2 Lymphocyte Doubling Time and Clinical Stage

Since the LDT measures the rate of expansion of the tumour clone and the clinical stage largely reflects the extent of the tumour burden it is not surprising that both these features predict for a shorter survival in CLL patients (Molica S *et al*, 2005). The stage of the disease, however, also reflects the distribution of tumour cells and those patients that predominantly have tumours cells located in lymph nodes or bone marrow have a poorer outcome and their tumours are more resistant to chemotherapy, than those

Table 1.1 – Features associated with clinical outcome in CLL

Disease Feature	Longer Survival	Shorter Survival
Gender	Female	Male
Age	Younger age	Older age
Clinical Stage	A	B or C
Lymphocyte Doubling Time	> 6 months	< 6 months
Family History	No family history	Family history of CLL
IGVH status	Mutated	Unmutated
CD38 expression	Low	High
Zap70 expression	Low	High
Cytogenetic Abnormalities	13q deletion or normal karyotype	17p deletion or 11q deletion
TP53 mutations	Wild type	Mutant

patients whose CLL cells are predominantly in a leukaemic phase (Binet JL *et al*, 1981, Rai KR *et al*, 1975, Molica S *et al*, 2005). This suggests that tumour cells have different characteristics according to their microenvironment.

It has been shown that CLL cells from the peripheral blood are non-cycling and are arrested in G1 of the cell cycle and typically these cells are characterised by an apoptotic defect *in vivo*. By comparison, CLL cells located within lymph nodes have been shown to cycle and proliferate, as indicated by the increased expression of the proliferation marker, Ki67 (Obermann EC *et al*, 2005). Leukaemic cell turnover has also been measured in CLL patients using heavy water (D_20), as a measure of the DNA synthesis in newly generated cells. This data has supported a two compartment model whereby tumour cells are produced in the proliferative compartment in lymph nodes and marrow and these are slowly released into the accumulative compartment in the peripheral circulation (Messmer BT *et al*, 2005).

These phenotypic differences between circulating tumour cells and those within the solid tissues are likely to relate to extrinsic signals from the microenvironment. Indeed, the apoptotic defect that is characteristic of CLL cells *in vivo* is known to be partly related to the acquisition of survival signals via interactions with non-tumour cells. These include the interaction of CLL tumour cells with T-cells, through CD40 ligand/CD40 receptor signalling, and with nurse-like cells and fibroblasts, through stromal derived factor 4 (SDF4) / chemokine receptor 4 (CXCR4) signalling. Furthermore, the stimulation of the CD40 receptor on CLL tumour cells by interaction with CD40 ligand has been shown to induce the expression of survivin, which appears to be important for the control of proliferation, and this is up regulated in tumour cells in the lymph

nodes and marrow (Ghia P *et al*, 2005, Munk Pedersen I, Reed J, 2004, Granziero L *et al*, 2001).

1.3.1.3 Family History

There is evidence for a genetic predisposition in the development of CLL and, interestingly, a positive family history of CLL has also been shown to be associated with certain adverse phenotypic characteristics.

A number of family pedigrees with multiple cases of CLL have been identified and recently population studies have confirmed that CLL is up to seven times more common in relatives of index cases than within controls (Neuland CY *et al*, 1983, Goldin LR *et al*, 2004). Further evidence to support the concept of a heritable basis underlying the development of a proportion of CLL cases has come through the detection of low-level clones of CD5+, CD23+ B-lymphocytes within normal individuals. Interestingly, these clones, which share genetic and phenotypic characteristics with indolent CLL tumours, occur over a younger age range in individuals that have a relative with CLL (Rawstron AC *et al*, 2002).

By comparison to sporadic cases of CLL, familial CLL has been shown to be associated with a younger age of onset and an increased incidence of complications including secondary malignancies (Ishibe N *et al*, 2001). In addition, in certain families the phenomenon of anticipation, such that the disease presents at younger ages in successive generations, has been reported (De Tute R *et al*, 2006).

1.3.2 Biological markers

A number of biological markers have also been previously identified that have the ability to predict clinical outcome in CLL (Table 1.1). The most well established of these have been confirmed in multiple patient series and include the mutation status of the immunoglobulin variable heavy genes (*IGVH*), the expression of the cell surface marker CD38, the level of expression of Zeta associated protein 70 (Zap70) and the presence of cytogenetic abnormalities (Hamblin TJ *et al*, 1999, Damle RN *et al*, 1999, Dohner H *et al*, 2000, Wiestner A *et al*, 2003).

1.3.2.1 Mutation status of the *IGVH* genes

The mutation status of the immunoglobulin heavy chain variable region gene (*IGVH*) was shown to provide prognostic information by two independent groups in 1999.

Patients were classified according to the degree of concordance between the sequences of the immunoglobulin heavy genes in their CLL tumour cells with the sequences of the nearest immunoglobulin heavy chain gene family. Those patients in which there was at least 98% concordance between the sequence of the *IGVH* genes in their tumour cells and the nearest family sequence were classified as having unmutated *IGVH* genes and those with less than 98% concordance were categorised as having mutated *IGVH* genes. The 98% cut-off level was used with the aim of distinguishing the group of patients whose tumour cells had not undergone the process of somatic hypermutation, but nevertheless had low level germ-line polymorphisms in their *IGVH* genes, from the group of patients whose tumour cells had acquired mutations in the *IGVH* genes as a consequence of somatic

hyper-mutation. Remarkably, those CLL patients with unmutated *IGVH* genes were shown to have a significantly shorter survival than those with mutated *IGVH* genes in their tumour cells (Figure 1.1) (Hamblin TJ *et al*, 1999, Damle RN *et al*, 1999).

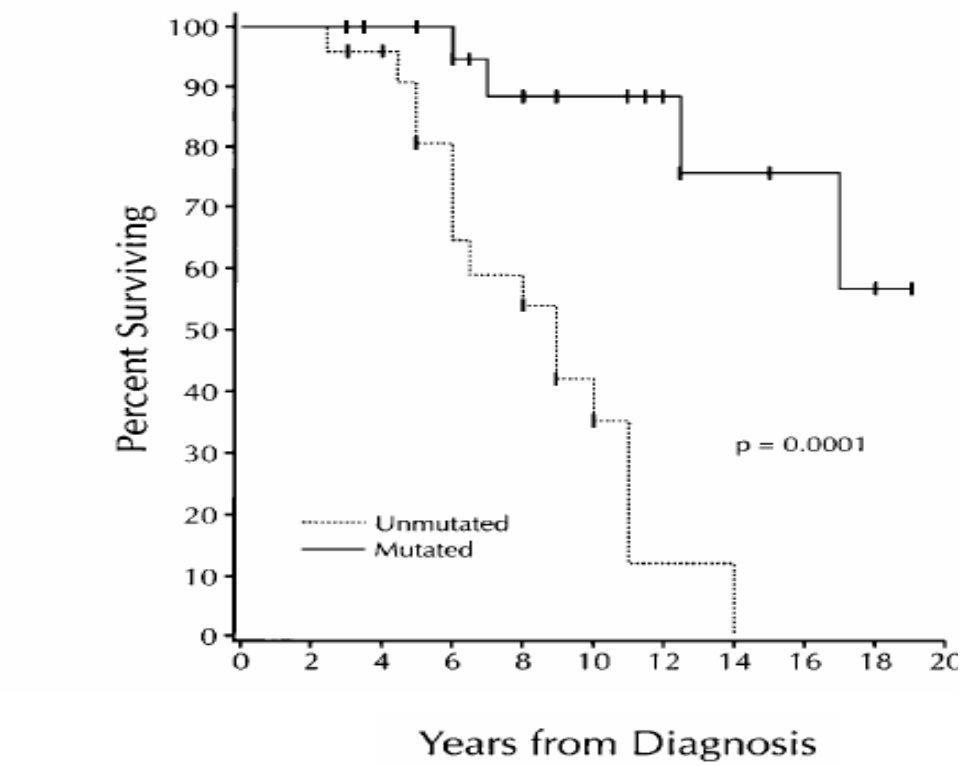
Somatic hypermutation of the *IGVH* genes in B-lymphocytes occurs in the response to antigen recognition during a T-cell dependent germinal centre reaction. Therefore, it was believed that CLL tumours with mutated *IGVH* genes had arisen from the clonal expansion of a B-lymphocyte that was antigen experienced and had undergone a germinal centre reaction. In contrast, tumours with unmutated *IGVH* genes were believed to have arisen from antigen naïve pre-germinal B-lymphocytes. It was hypothesised that the differing nature of the cell that had become clonally transformed might account for the differences in the tumour behaviour and consequently the clinical outcome of the two subgroups of CLL patients (Stevenson F *et a*l, 1998).

More recent data has suggested that the relationship between *IGVH* mutations status, clinical outcome and cellular origin may be more complex in CLL than previously thought. Gene expression profiling has provided data suggesting that both *IGVH* unmutated and mutated CLL tumours closely resemble memory B-cells with only minimal differences (Rosenwald A *et al*, 2001, Klein U *et al*, 2001). One interpretation to account for these data is that all CLL tumours have arisen from the transformation of antigen-experienced lymphocytes but there may be differences in the nature of the antigen and the type of response (Klein U *et al*, 2001). Furthermore, certain immunoglobulin gene families, such as *VH3-21*, have been shown to result in poor clinical

Figure 1.1

The mutation status of the *IGVH* gene was identified as an important prognostic marker in CLL patients in 1999 (Hamblin TJ *et al*, 1999, Damle RN *et al*, 1999). Patients whose tumour cells had un-mutated *IGVH* genes were shown to have a shorter survival from diagnosis than patients whose tumour cells carried mutated *IGVH* genes. These differences were highly statistically significant.

Figure 1.1



Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK (1999).
Unmutated IgVH genes are associated with a more aggressive form of chronic lymphocytic leukaemia.
Blood; **94**(6):1848-1854

outcome regardless of the *IGVH* mutation status and, interestingly, it has also been found that the association of different variable (V), diversity (D) and junctional (J) immunoglobulin gene segments occurs in a non-random manner in CLL tumours (Tobin G *et al*, 2003, Stevenson FK, Caligaris-Cappio F, 2004). Therefore, it is now believed that the differential outcome in patients according the *IGVH* mutation status may be related to the persistence of antigen recognition and consequent signalling through the B-cell receptor (BCR) on the tumour cells, which could convey survival signals. These antigens might include self-antigens and they may be more readily recognised by tumours with unmutated *IGVH* genes, because of the lower stringency for antigen recognition by their BCRs. Therefore, this could account for the poorer outcome in these CLL subgroups (Stevenson FK, Caligaris-Cappio F, 2004).

Thus it can be seen that the discovery of the prognostic value of the *IGVH* mutation status has significantly increased the understanding of the nature of CLL. One advantage of the status of the *IGVH* genes as a biological marker is that it is determined at the time of clonal transformation and is largely stable over time. A disadvantage of the *IGVH* mutation status is that it is not always reliable at predicting outcome for an individual patient and, notably, a significant proportion of patients with mutated *IGVH* genes will still have an aggressive disease phenotype (Hamblin TJ *et al*, 1999, Damle RN *et al*, 1999). In addition, there have also been discussions regarding the appropriate cut-off to define tumours with mutated and un-mutated *IGVH* genes. Certain groups have used a 95% cut-off to define mutated and unmutated *IGVH* genes on the basis of the frequency of mutations in the *IGVH* gene in non-

clonal CD5 positive B-lymphocytes (Lin *et al*, 2002). However in this study patients with greater than 98% concordance with the respective *IGVH* family sequence still had the shortest survival, but patients with 95-98% concordance had a poorer outcome than patients with <95% concordance (Lin *et al*, 2002).

Nevertheless, the mutation status of the *IGVH* genes has remained a gold standard for the evaluation of new biological prognostic markers in CLL.

1.3.2.2 CD38 expression

The *IGVH* mutation status is technically difficult to perform and is not generally available in diagnostic laboratories and there has therefore been interest in finding alternative markers that correlate with the mutation status of the *IGVH* genes.

The level of expression of the activation marker Cluster of Differentiation 38 (CD38) was found to provide prognostic information in CLL and also to correlate with *IGVH* mutation status (Damle RN *et al*, 1999, Ghia P *et al*, 2003). Patients whose tumours had unmutated *IGVH* genes were found to have elevated CD38 expression and also to have a poorer clinical outcome in comparison to those with low levels of CD38 expression on their tumour cells.

Subsequently, the correlation between these two prognostic markers was shown to be incomplete, although both markers provide independent prognostic information (Vilpo J *et al*, 2003). In addition, it was also found that certain patients have populations of tumour cells exhibiting bimodal patterns of CD38 expression. The clinical consequences of this bimodal CD38 expression have not been fully resolved however there is some evidence to

suggest that they do not imply a poorer survival (Ghia P *et al*, 2003, Cocco AE *et al*, 2005). Interestingly, it has also recently been reported that CD38 expression can be induced in CLL cells in certain *in vitro* culture systems. This suggests that its level of expression might be related to extrinsic signalling factors from the microenvironment, rather than representing an inherent feature of the tumour cells (Pepper C *et al*, 2006, Ghia P *et al*, 2003).

Despite these considerations, CD38 remains a useful prognostic marker that is commonly used in the clinical setting because it is readily quantifiable by immunophenotyping using a fluorescent activated cells sorting (FACS) assay and because its expression level provides information that can predict clinical outcome.

1.3.2.3 Zap-70 expression

The zeta-associated protein 70 (Zap70) was identified as another potential surrogate marker for *IGVH* mutation status following gene expression profiling studies (Wiestner A *et al*, 2003). Elevated Zap70 expression was found to predict outcome in CLL patients and to be associated with unmutated *IGVH* genes, although once again this correlation is incomplete (Rosenwald A, 2001, Kim SZ *et al*, 2004, Wiestner A *et al*, 2003).

The mechanism through which elevated Zap70 conveys a poor prognosis in CLL patients may relate to its role in the transduction of signals from the BCR on tumour cells. Such signalling could favour the induction of an anti-apoptotic phenotype and hence a poorer clinical outcome (Chen L *et al*, 2005). Interestingly, in an analogous manner to CD38 status there is also

evidence to suggest that the level of expression of Zap70 can vary within an individual and that it is also inducible in *in-vitro* culture systems. This again suggests that its expression level may not be a stable feature of the tumour clone (Cocco AE *et al*, 2005, Pepper C *et al*, 2006).

Zap70 expression on clonal B-cells can also be quantified using FACS analysis, however difficulty in the development of reproducible assays to reliably quantitate its expression has so far impeded its widespread use as a prognostic marker in the clinical setting (Wiestner A *et al*, 2003).

1.3.2.4 Cytogenetic abnormalities

Several recurrent chromosomal aberrations have been described in CLL tumours and these have also been found to provide prognostic information and may therefore identify specific subgroups of CLL patients whose outcome is more homogeneous. The most frequent changes that have been detected are deletions of chromosomes 13q, 11q, 17p and 6q and trisomy of chromosome 12 (Dohner H *et al*, 2000).

A deletion of chromosome 13q is a common finding and occurs in up to 80% of CLL tumours. When present as an isolated change it is associated with a good clinical outcome which is similar to that observed in patients with a normal karyotype. No tumour suppressor gene candidate has been established on the region of deletion on chromosome 13q but, interestingly, two micro-RNA (mirs) genes, mirs15 and 16, have recently been identified. These have been shown to regulate the expression of the anti-apoptotic protein BCL-2, which is known to be elevated in B-CLL tumour cells, thus

providing a potential mechanistic advantage for the loss of chromosome 13q in CLL tumour cells (Croce C *et al*, 2005).

The predictive value of both trisomy of chromosome 12 and a deletion of chromosome 6q remains uncertain but each may have a small impact on patient outcome. These defects occur in CLL tumours at a frequency of up to 16% and 7%, respectively (Dohner H *et al*, 2000; Dewald GW *et al*, 2003).

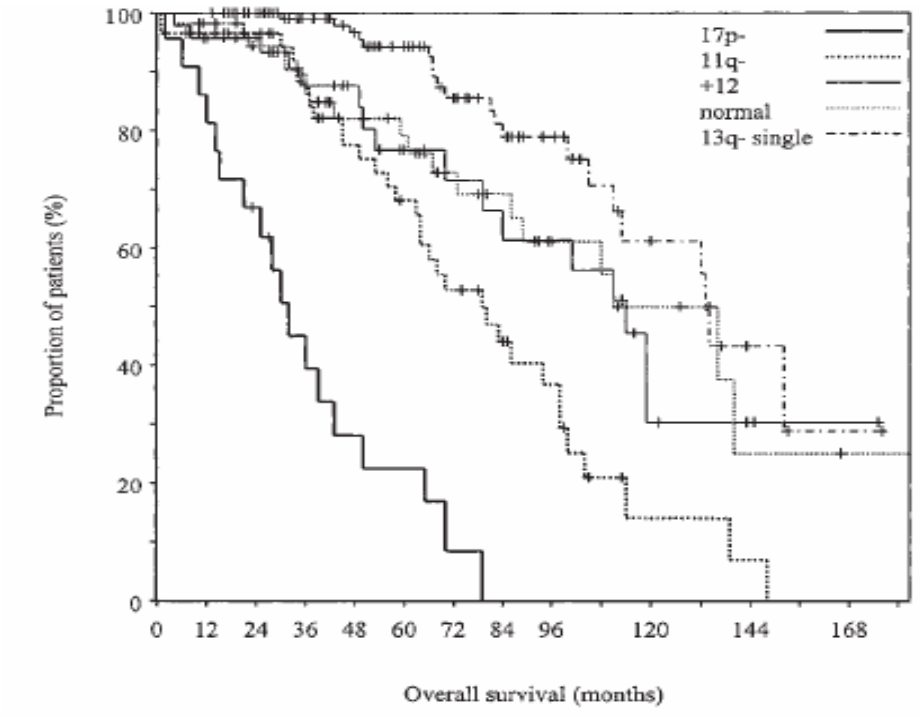
By comparison, deletions of chromosomes 11q and 17p have been shown to result in a significantly shorter survival in CLL patients in comparison to patients with a normal karyotype or an isolated 13q deletion (Dohner H *et al*, 2000). Since both markers are associated with an aggressive disease phenotype their prevalence will vary according to the nature of the cohort. For example, their frequency has been shown to occur in 9 and 4%, respectively, of stage A patients compared to 20 and 5% of patients requiring first line treatment (Seiler T *et al*, 2006). Patients with a deletion of 17p have the shortest survival and patients with an 11q deletion have an outcome that is intermediate between those with a normal karyotype or 13q deletion and those with a 17p deletion (Dohner H *et al*, 2000). Deletions of chromosome 11q have also been shown to be associated with both extensive lymphadenopathy and unmutated *IGVH* genes in CLL patients and deletions of 17p have been found to predict resistance to chemotherapeutic agents (Dohner H *et al*, 1997, Sturm I *et al*, 2003) (Figure 1.2).

The minimal region of deletion on chromosome 17p includes the *TP53* gene that encodes for the pro-apoptotic protein p53. Loss of one *TP53* allele, in combination with a *TP53* mutation on the second allele, will lead to impaired p53 function and this combination results in an apoptotic resistant

Figure 1.2

Cytogenetic abnormalities have been shown to provide prognostic information in CLL patients. Several recurrent abnormalities have been identified, namely deletions of chromosomes 13q, 11q, 17p and 6q and trisomy of chromosome 12. Patients with a deletion of chromosomes 17p or 11q have been shown to have a shorter survival than patients with either a normal karyotype or the presence of an isolated chromosome 13q deletion or trisomy 12. The shortest survival is seen in patients with a 17p deletion and patients with an 11q deletion have a survival that is intermediate between those with a 17p deletion and those with a normal karyotype (Dohner H *et al*, 1999).

Figure 1.2



Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, Dohner K, Bentz M, Lichter P (2000). Genomic aberrations and survival in chronic lymphocytic leukaemia.
N Engl J Med; **343**(26):1910-1916

phenotype (Stankovic T *et al*, 2002). This defect in apoptosis is believed to be the mechanism accounting for the very poor prognosis in these patients. The detection of a deletion of chromosome 17p appears to be a powerful predictor of poor outcome for the individual patient and as a result may directly influence treatment decisions, particularly in young patients (Binet JL *et al*, 2006).

The minimal region of deletion on chromosome 11q has been previously delineated and shown to consist of a sequence of between 2-3 megabases that includes a number of genes, one of which is the *ATM* gene (Stilgenbauer S *et al*, 1996). The *ATM* gene has, therefore, been identified as a putative tumour suppressor gene that might be associated with the poor outcome in this subgroup of CLL patients. The role of abnormalities in the *ATM* gene and their potential effects on the function of the ATM protein is the subject of this thesis.

These recurrent chromosomal aberrations can now be readily detected with a high sensitivity using interphase fluorescent in situ hybridisation (FISH) in CLL tumours. *TP53* and *ATM* probes are used for the detection of chromosome 17p and 11q deletions and therefore any tumour found to have one of these deletions by FISH will, by definition, have lost one allele of the respective gene (Dewald GW *et al*, 2003). The detection of these chromosomal defects is increasingly being used in the clinical setting to identify those subgroups of patients that will have a poorer outcome and in the future may allow the targeted use of new therapeutic approaches.

1.4 The ATM protein

The *ATM* gene, located on chromosome 11q22-23, encodes for the 370kD ATM protein. Individuals that inherit two germline mutations in the *ATM* gene have the disorder ataxia telangiectasia (AT) (Taylor AM, Edwards MJ, 1982). The clinical and cellular phenotypes of AT individuals have provided a means to study the *ATM* gene and to understand the functions of the ATM protein. The increasing understanding of the biological function of ATM continues to facilitate the study of the role of abnormalities in the *ATM* gene in the pathogenesis of CLL and other lymphoid tumours.

1.4.1 The *ATM* gene

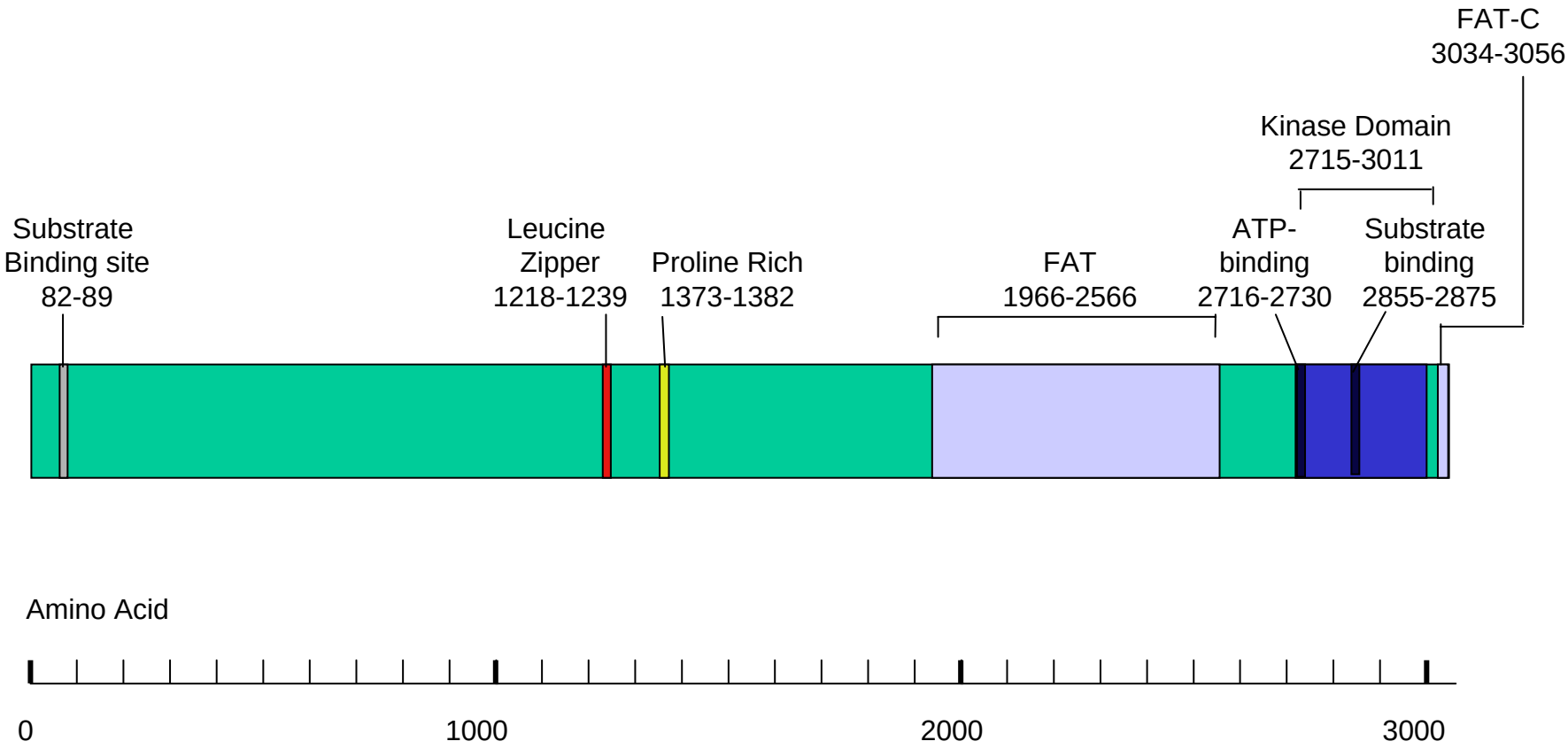
The *ATM* gene has 65 exons extending over 160kb of genomic DNA and gives rise to a 13kb transcript which has an open reading frame of 9.138 kb. The ATM protein is a member of the phosphoinositide 3-kinase-like kinases (PIKK) family and shares sequence homology with other members of this group that includes DNA protein kinase catalytic sub-unit (DNA-PKcs), Ataxia Telangiectasia related protein (ATR), mammalian target of rapamycin (mTOR) and the Transactivation-transformation domain associated protein (TRRAP). The characteristic feature of this family of proteins is the phosphorylation of target proteins on serine and threonine residues, which are recognised as SQ (serine/glutamine) or TQ (threonine/glutamine) motifs (Shiloh Y, 2003).

A number of functional, or putatively functional, domains have been identified in the *ATM* gene sequence (Figure 1.3). The catalytic kinase domain is present at the C-terminal end of the gene and is highly conserved across species as well as between related family members. Within the translated

Figure 1.3

Cartoon of the 370 kilo-dalton ATM protein that demonstrates the position of the functional domains. These include the catalytic kinase domain, which is located from amino acid residues 2715 to 3011 and itself includes an ATP binding site (residues 2716 and 2730) and a catalytic subunit (residues 2855 and 2875). The FAT and FATc domains represent regions of close sequence homology between related proteins and extend from residues 1966 to 2566 and from 3034 to 3056, respectively. Additional domains include a substrate-binding domain at the n-terminus of the gene and a putative leucine zipper and proline rich domain located from residues 1218 to 1238 and from 1373 to 1382, respectively.

Figure 1.3


FAT-C
3034-3056
Kinase Domain
2715-3011
Substrate
Binding site
82-89
Leucine
Zipper
1218-1239
Proline Rich
1373-1382
FAT
1966-2566
ATP-
binding
2716-2730
Substrate
binding
2855-2875
Amino Acid
0
1000
2000
3000

ATM protein, this domain extends from amino acid residues 2715 to 3011 and includes an ATP binding site located between residues 2716 and 2730 and a catalytic subunit located between residues 2855 and 2875.

Two further domains that have been described in the *ATM* gene are the FAT domain and the FATc domain. The FAT domain extends from residues 1966 to 2566 in the ATM protein and represents a region that is closely conserved between related proteins. The FATc domain is also conserved in PIKK proteins and is located at the extreme C terminal of the protein from residues 3034 to 3056. The FAT and FATc domains are believed to fold together in a configuration that ensures efficient function of the kinase domain (Lavin M *et al*, 2003). In addition, the FATc domain contains a substrate-binding domain, which is involved in the interaction with the Tip60 histone acetyltransferase (Jiang X *et al*, 2006). This interaction has recently been reported to be important for the activation of ATM (Sun Y *et al*, 2005).

Towards the middle of the ATM protein are two further domains that may have a putative function. Firstly, an incomplete leucine zipper from residues 1218 to 1238 has been detected, which theoretically could facilitate protein-protein interactions through the outward extension of the leucine residues, although no such interaction has been identified to date. Secondly, a proline rich region, from residues 1373 to 1382, has been described and has been implicated in the binding of ATM to the SH3 domain of the c-Abl tyrosine kinase. In addition, a further substrate-binding domain has been mapped to the N-terminus of the ATM protein from residues 82 to 89, which is believed to be important for the binding of p53, BRCA1 and BLM (Lavin M *et al*, 2003).

The function of the majority of the remaining sequence is poorly understood but may be mainly related to the formation of the tertiary structure of the ATM protein within the cell. Numerous HEAT (*H*untington, *e*longation factor 3, *A* subunit of protein phosphatase 2A and *T*OR1) repeats have been identified within the N-terminal two thirds of the protein, and there is preliminary evidence to suggest that these allow the ATM protein to fold to form a coiled 'head structure' whereas the C-terminal portion of the protein including the catalytic domain forms a 'tail structure'. Indeed, electron microscopy has directly visualised the protein in the cell and supports this tertiary structure of the ATM protein (Llorca O *et al*, 2003).

1.4.2 Clinical Phenotype of AT patients

AT is a rare neurodegenerative disorder that is characterised by a progressive cerebellar ataxia and the development of dermal and ocular telangiectasia. Patients with 'classical AT' have no ATM protein in their cells and in these cases the ataxia is usually obvious by the age of 2 years and individuals will typically be unable to walk and will require a wheelchair by their early teens The life expectancy of patients with classical AT is approximately 20-25 years (Lakin ND; 1996, Taylor AM, Byrd PJ; 2005, Crawford TO, 2006).

AT patients also have primary immunodeficiency that results in an increased likelihood for the development of severe infections. Although there is a degree of heterogeneity between individuals, AT patients typically have impairment of both T and B-cell immunity. For example, there is impaired activity of CD4-helper and CD8-cytotoxic T-lymphocytes together with a

poorly developed or absent thymus in most AT individuals. The majority of AT patients also have reduced IgA, IgG2 and IgE levels, suggesting that they have a defect in the immunoglobulin isotype class switch process (Waldmann TA *et al*, 1983).

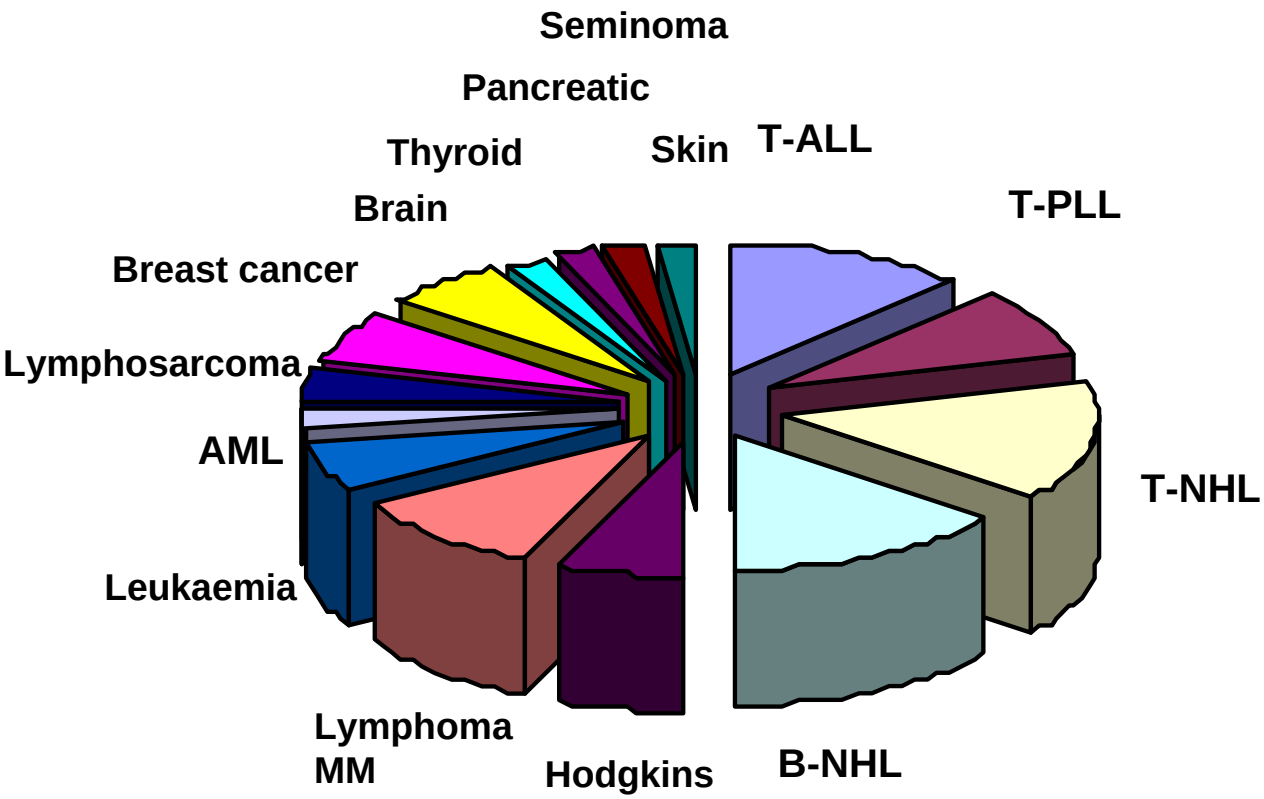
The T-lymphocytes in A-T patients are further characterised by the presence of chromosomal translocations which frequently involve the sites of the T cell receptor (TCR) genes on chromosomes 7 and 14. These occur at an approximately 40-fold higher frequency in AT patients compared to non-AT individuals. In certain cases, where the break-point on the partner chromosome involves an oncogene, cells harbouring these translocations can proliferate to form large clones. Furthermore, there is evidence that the formation of these clones can precede the development of the malignant tumour, T-cell prolymphocytic leukaemia (T-PLL), in some AT patients (Taylor AMR *et al*, 1992). Sporadic T-PLL is a rare tumour but the frequency of T-PLL is markedly increased in AT patients and also occurs over a younger age range. Therefore, the development of these chromosomal translocations is likely to be important in the pathogenesis of this leukaemia.

AT patients have a markedly increased frequency of all lymphoid tumours which is approximately 200-fold higher than in the general population. The spectrum of haematological malignancies seen in AT patients is shown in Figure 1.4. The most common types of tumours are T-cell lymphomas and leukaemias, including T-PLL. However, there is also a clear increase in the frequency of B-cell lymphomas in these AT individuals. Interestingly, there is some evidence to suggest that chromosomal translocations occur in B-lymphocytes, through the breakage and fusion of

Figure 1.4

Patients with ataxia telangiectasia have a marked increase in the development of multiple types of cancers. The spectrum and frequency of tumours that develop in these individuals is shown. Haematological malignancies account for over two thirds of the tumours and the most common types are T-cell tumours including T-PLL and T-ALL. Notably these patients also have an increase in the development of B-cell lymphoid tumours.

Figure 1.4


Seminoma
Pancreatic
Thyroid
Skin
T-ALL
Brain
T-PLL
Breast cancer
Lymphosarcoma
AML
T-NHL
Leukaemia
Lymphoma
MM
Hodgkins
B-NHL

two immunoglobulin genes, in an analogous manner to the TCR rearrangements in T lymphocytes. Small clones of B-lymphocytes containing these translocations have been described at an increased frequency in AT patients compared to normal individuals, and they may contribute to the increased frequency of B–cell tumours (Taylor AM, et al; 1996).

1.4.3 Cellular phenotype of AT cells

The cells from AT patients characteristically have a radio-sensitive phenotype and the demonstration of this radio-sensitivity is a requirement in order to make the diagnosis of AT. Following treatment with irradiation, AT cells have an increased number of unrepaired DNA double strand breaks (DNA DSBs) compared to control cells and this in turn leads to the formation of chromosomal aberrations that include tri-radicals and quadri-radicals as well as translocations (Taylor AM *et al*, 1975). The specific repair defect in AT cells has subsequently been characterised as the persistence of those double strand DNA breaks that are difficult to resolve, typically accounting for around 10% of DNA breaks. Interestingly, the kinetics of the repair of the other DSBs is relatively unaffected in AT cells, suggesting that ATM may not be important in the resolution of these simpler lesions (Riballo E *et al*, 2004).

1.4.4 Role of ATM in the DNA damage response

The observations of the clinical features of AT patients and the radiosensitive phenotype of AT cells have provided many clues to our current understanding of the function of the ATM protein. The principal role of ATM is now known to be the rapid coordination of an integrated cellular response to

the presence of double strand DNA breaks. This leads to activation of multiple downstream proteins, which in turn result in the arrest of the cell cycle, and either the repair of the DNA DSBs or the induction of apoptosis, if the damage is extensive or difficult to resolve (Shiloh Y, 2003).

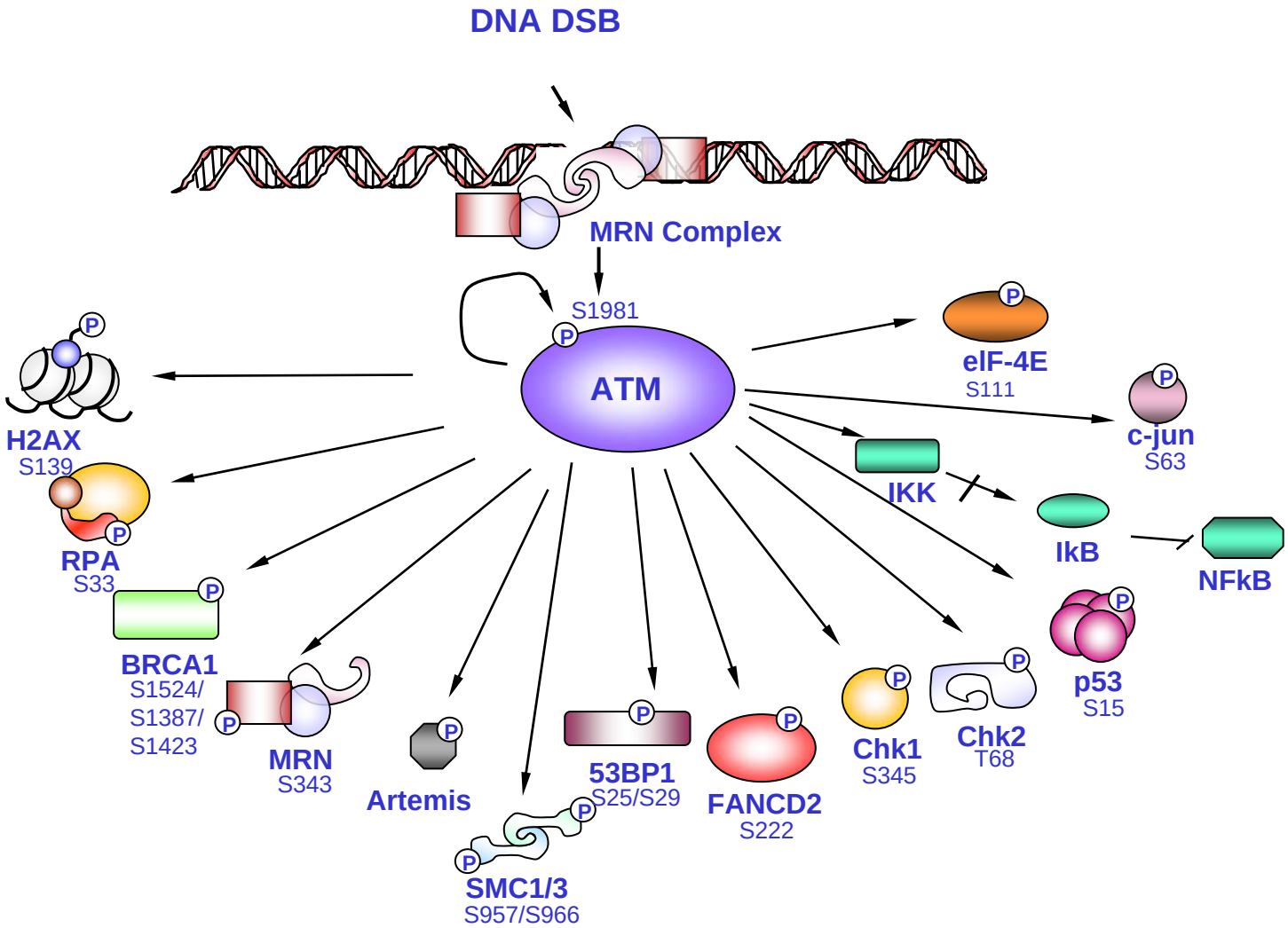
As a member of the PI3 kinase–like kinase family of proteins, ATM mediates its function through the phosphorylation of serine and threonine residues on target proteins. A large number of target substrates have now been identified and the activity of these substrates can be rapidly altered by ATM dependent phosphorylation (Shiloh Y, 2003, Goodarzi A *et al*, 2003). The spectrum of target proteins is shown in Figure 1.5.

ATM is no longer believed to be the principal sensor of DNA DSBs, and there is evidence that it is recruited to the sites of breaks by proteins acting upstream in the damage response pathway. A complex of proteins termed the MRN complex that consists of the proteins Mre11, Nijmegen breakage syndrome 1 (Nbs1) and Rad50 appears to be essential for this rapid recruitment of ATM (Petrini JH, Stracker TH, 2003). In the resting cell ATM exists as a dimer or multimer and its activation at the sites of the DNA breaks involves a process of auto-phosphorylation, which leads to the formation of ATM monomers (Bakkenist CJ, Kastan MB, 2003). The activation of ATM may also involve its acetylation via an interaction with Tip-60 (Sun Y *et al*, 2005) Active monomeric ATM can phosphorylate multiple downstream proteins. Interestingly, the MRN proteins Nbs1 and Mre11 are themselves phosphorylated in an ATM-dependent manner after DNA damage, suggesting that the MRN complex and ATM function both upstream and downstream of

Figure 1.5

ATM is a protein kinase that alters the function of target proteins through the phosphorylation of specific serine or threonine residues. ATM coordinates an integrated cellular response following the induction of DNA double strand breaks. The spectrum of targets that become phosphorylated by ATM after this type of DNA damage is shown. These include proteins involved in DNA repair, such as Brca1, proteins important for the induction of cell cycle arrest, including p53, Chk1 and Chk2, and proteins important for the induction of apoptosis such as p53.

Figure 1.5


DNA DSB
MRN Complex
S1981
ATM
H2AX
S139
RPA
S33
BRCA1
S1524/
S1387/
S1423
MRN
S343
Artemis
53BP1
S25/S29
SMC1/3
S957/S966
FANCD2
S222
Chk1
S345
Chk2
T68
p53
S15
IKK
IkB
NFkB
eIF-4E
S111
c-jun
S63
P

each other as the recognition process of DSBs proceeds (Kurz EU, Lees-Miller SP, 2004).

1.4.4.1 Formation of γH2AX foci

The histone variant H2AX is another substrate that becomes rapidly activated by ATM after the formation of DSBs. Phosphorylated H2AX, termed γH2AX, can be observed over several megabases flanking the DSBs at short times after irradiation (Rogakou EP *et al*, 1999). It is believed that γH2AX has a critical role in facilitating the assembly of large DNA repair complexes on the damaged DNA. Additional proteins including MDC1, 53BP1, the MRN complex of proteins and BRCA1 accumulate to form large foci at the sites of H2AX phosphorylation. There is evidence suggesting that the proteins 53BP1 and MDC1 are also required for the activation of ATM and they are believed to act as adaptor proteins for the recruitment of further ATM target proteins to the sites of the breaks (Goldberg M *et al*, 2003, Stewart GS et al, 2003, Mochan TA *et al*, 2003, Foray N *et al*, 2003). These target proteins include DNA repair proteins, such as Ku70/80, DNAPKcs, XRCC4, Ligase IV or the Rad51 complex, and also proteins responsible for activation of cell cycle checkpoints and apoptosis, such as p53, Chk2, and SMC1 (Kurz EU, Lees-Miller SP, 2004, Riballo E, *et al*; 2004).

1.4.4.2 Redundancy of certain functions of the ATM protein

ATM has been described as the master regulator of the response to DNA DSBs due to its large number of target substrates and its role in influencing different cellular pathways, (Kurz EU, Lees-Miller SP, 2004). However, ATM is

not an absolute requirement in the cell, since ATM knock-out mice are viable and AT patients, with no demonstrable ATM function, are able to survive for up to 25 years (Dantzer F, 1999, Taylor AM, 2005). This suggests that there must be some redundancy between the function of ATM and related proteins. Related members of the PIKK family, including ATR and DNA-PKcs, can compensate for certain functions of the ATM protein (Koundrioukoff S *et al*, 2004). For example, DNA-PKcs is known to be particularly important for the phosphorylation of substrates that are involved in the repair of DNA DSBs but recently it has been also been shown that DNA-PKcs can function in a redundant manner with ATM, in the process of phosphorylation of H2AX after the induction of DSBs (Alt F, Bassing C, 2003, Stiff T *et al*, 2004). ATR is considered to be principally involved in the response to UV light-induced DNA damage but it has also been shown to be involved in the phosphorylation of several substrates including p53 following DNA damage with IR (Cuadrado M *et al*, 2006, Helt CE *et al* 2005).

Nevertheless, the compensation between these proteins cannot be complete given the known clinical phenotype of AT patients, and it is likely that both the target substrate specificity and the kinetics of the response to DSBs are important distinguishing factors between ATM and both DNA-PK and ATR. Indeed, a unique aspect of ATM appears to the very rapid response to DNA DSBs (Helt CE *et al* 2005, Myers JS et al, 2006).

1.4.5 ATM dependent activation of p53 after DNA damage

One downstream substrate that is phosphorylated on multiple residues by ATM, both directly and indirectly, following the occurrence of DSBs is the

tumour suppressor protein p53. These changes lead to the up regulation of p53 levels in the cell nucleus. The p53 protein has both transcription-dependent and transcription-independent activities and characteristically undergoes a wide range of post-translational modifications (Meek D, 2004, Vousden KH, 2002). There is evidence to suggest that p53 plays a critical role in determining the final cell fate after DNA damage. Indeed, p53 appears to be a crucial factor for the switch between the induction of cell cycle arrest, thus allowing DNA repair, and the induction of apoptosis which may occur in the presence of extensive or persistent DNA damage (Bree RT *et al*, 2004).

These differential functions of p53 may be executed through variations in post-translational modifications. For example, ATM dependent phosphorylation of serine 15 has been shown to enhance the transcriptional activity of p53 and also to promote the phosphorylation of additional p53 residues. In comparison the ATM-Chk2 mediated phosphorylation of p53 on serine 20 interferes with p53 binding to the MDM2 protein, which is required for targeting p53 for proteasomal degradation in the cytoplasm (Bree RT *et al*, 2004). The phosphorylation of p53 on other residues can influence its binding to specific gene promoters and, interestingly, there is evidence suggesting that the phosphorylation on serine 46, which is induced by high levels of stress, can significantly influence the outcome of the p53 pathway in favour of apoptosis (Oda K *et al*, 2000). Thus lower levels of stress may lead to alterations in p53 that favour the induction of expression of cell cycle check point proteins, such as p21, whereas high levels of stress occurring when there is extensive or persistent DNA damage would favour alterations that

induce the expression of pro-apoptotic proteins, such as Puma and Bax (Bree RT *et al*, 2004) (Meek D, 2004).

1.4.6 Repair of DNA double strand breaks

The two major mechanisms that have evolved for the repair of DNA DSBs in mammalian cells are homologous recombination (HR) and non-homologous end joining (NHEJ). HR is an error-free repair mechanism but is restricted to the S and G2 phases of the cell cycle as there is a requirement for the presence of sister chromatids. It may therefore be less important in the repair of DNA DSBs in non-cycling CLL cells.

NHEJ is an error-prone repair pathway and is the major mechanism used in the repair of DNA DSBs in non-cycling cells. The principal proteins involved are Ku70 and 80, DNA-PKcs, Artemis, XRCC4 and DNA ligase 4. The Ku proteins bind to the broken DNA ends and this leads to the subsequent recruitment of DNA-PKcs, which has a role in bridging the broken ends together. Artemis is important in the processing of the DNA ends prior to their ligation, which is catalysed by the XRCC4 and DNA ligase 4 proteins (Bassing CH, Alt FW, 2003). Difficulty in the processing of complex DNA breaks may lead to their persistence and this is one reason that will influence the switch in cell fate towards apoptosis. As alluded to in the text above, ATM itself also has a role in the repair of certain complex DNA DSBs by NHEJ (Riballo E *et al*, 2004).

1.4.7 ATM dependent DNA damage induced apoptosis

If the DNA damage cannot be repaired then the cell activates programmed cell death or apoptosis. The persistence of DNA DSBs would lead to genomic instability and therefore even a single unrepaired DSB may be lethal.

The process of DNA damage-induced apoptosis occurs in a p53 dependent manner. Through its function as a transcription factor, p53 leads to the induction of certain pro-apoptotic proteins, including members of the BcL-2 family of proteins such as Puma, Noxa and Bax. This will alter the balance between the various pro- and anti- apoptotic members of this family of proteins. Pro-apoptotic proteins in this family also include the BH3-domain only proteins, such as Bid and Bim, and anti-apoptotic family members include Mcl-1, BCL-xL and Bcl-2. Alterations in the interactions between these related proteins on the surface of the mitochondrion, leads to the depolarisation of the mitochondrial membrane. This in turn results in the release of cytochrome C from the inter-membrane mitochondrial space into the cytoplasm. Here, cytochrome C associates with the apoptosis-activating factor (apaf), ATP and procaspase 9 to form the apoptosome structure. This subsequently leads to the cleavage of initiator procapases, such as procaspase 7. Cleaved caspase 7 can then catalyse the cleavage of the procaspase 3 into its active cleaved caspase counterpart. Caspase 3 is an effector caspase that can cleave a large number of downstream targets and thus induce the cascade of events that eventually results in the completion of the apoptotic process (Herr I, Debatin K-M, 2001, Yee KS, Vousden K, 2005, Tsujimoto Y, 1998).

A summary of the ATM/p53 dependent DNA damage response apoptotic pathway is shown in Figure 1.6.

1.5 The role of *ATM* mutations in the pathogenesis of CLL tumours

Mutations in the *ATM* gene have been previously detected in CLL tumours (Stankovic T *et al*, 1999, Schaffner C *et al*, 1999, Bullrich F *et al*, 2000). Therefore, it is possible that they could represent a molecular marker that might provide prognostic information in CLL patients. Furthermore, given the known functions of the ATM protein in the response to DNA damage, it is likely that abnormalities in ATM will provide important insights into the pathogenesis of CLL tumour clones.

1.5.1 The discovery of *ATM* mutations in T-PLL and CLL tumours

The presence of mutations in the *ATM* gene was first described by independent groups in 1999 and 2000(Stankovic T *et al*, 1999, Schaffner C *et al*, 1999, Bullrich F *et al*, 2000). At that time, there were several lines of evidence to suggest that the *ATM* gene was a candidate gene that might harbour abnormalities in CLL. Firstly, a deletion of chromosome 11q was known to be a common chromosomal aberration in CLL tumours and the minimal region of deletion had been delineated and had been shown to include the *ATM* gene (Stilgenbauer S *et al*, 1996).

Furthermore, acquired *ATM* mutations had been reported to occur at a high frequency in the rare lymphoid tumour T-cell pro-lymphocytic leukaemia (T-PLL). Similarly to CLL, sporadic T-PLL is also characterised by a high

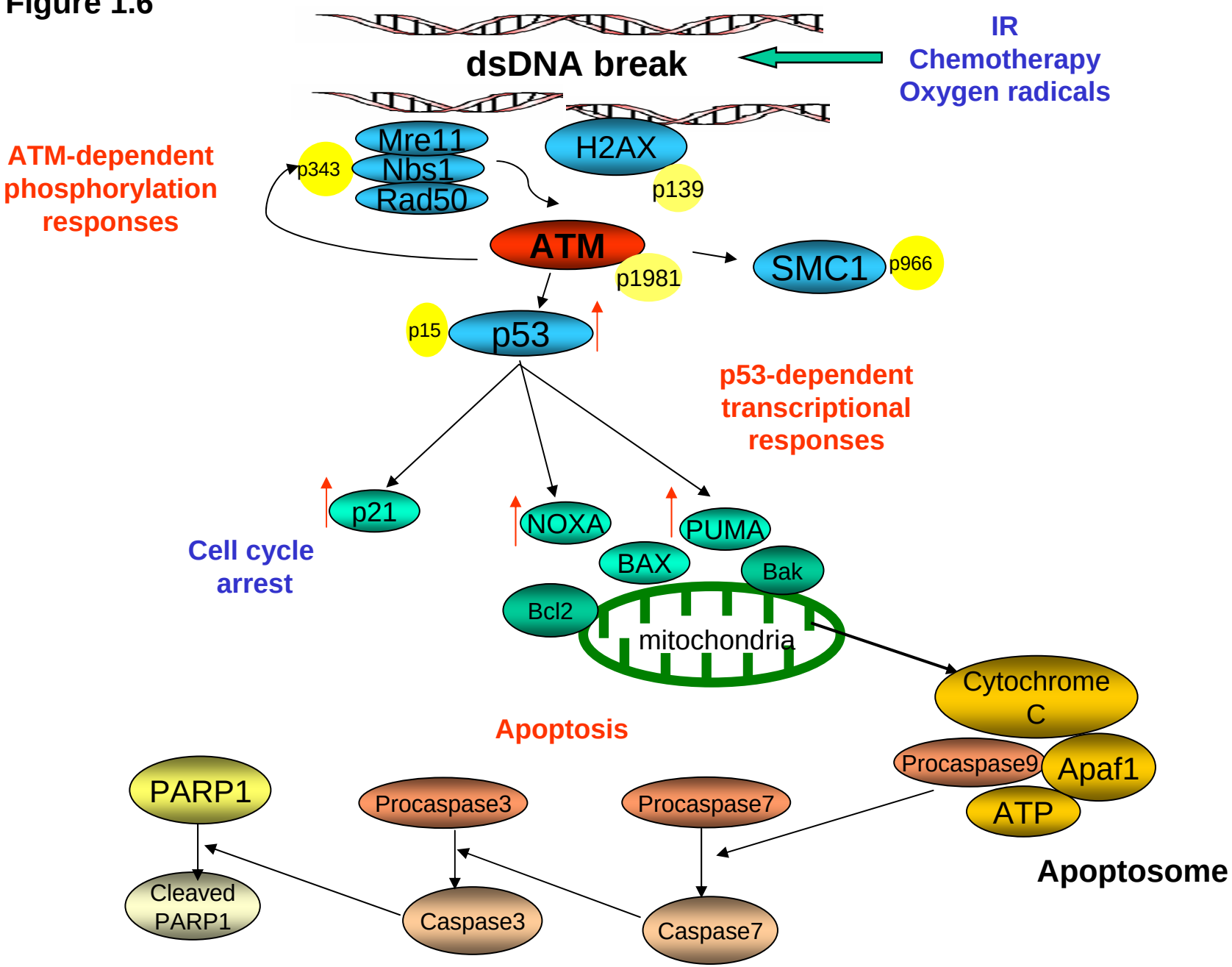
Figure 1.6

The ATM / p53 pathway that is activated following the induction of DNA damage is demonstrated. DNA DSBs can occur following treatment with irradiation or chemotherapy or through oxidative damage. The ATM protein is recruited to the sites of the breaks via the MRN complex and becomes activated by autophosphorylation on serine 1981. It then activates various downstream target proteins, including H2AX, which is important for the formation of large foci at the sites of the DNA breaks, and SMC1, p53 and Nbs1.

The phosphorylation of p53 by ATM on serine 15 leads to its up regulation in the nucleus where it can act as a transcription factor. This in turn leads to the expression of various proteins that are important for the induction of cell cycle arrest, such as p21, or apoptosis such as Puma, Noxa and Bax. In the presence of extensive or persistence DNA damage signaling through this pathway will lead to apoptosis.

Apoptosis principally occurs through the intrinsic pathway, which is demonstrated. Alterations in the balance between pro-apoptotic BH3 domain proteins such as Bax, Noxa and Puma and anti-apoptotic proteins such as Bcl-2 and Mcl-1 leads to depolarization of the mitochondrial membrane and the release of cytochrome c from the intermembrane space into the cytoplasm. Here it forms the apoptosome structure, which eventually results in the cleavage of the effector procaspase 3. Cleaved caspase 3 can then inactivate numerous cellular proteins via a cleavage mechanism, including PARP1, which facilitate apoptosis.

Figure 1.6


dsDNA break
IR
Chemotherapy
Oxygen radicals
ATM-dependent phosphorylation responses
Mre11
Nbs1
Rad50
p343
H2AX
p139
ATM
p1981
SMC1
p966
p15
p53
p53-dependent transcriptional responses
p21
NOXA
PUMA
BAX
Bak
Bcl2
mitochondria
Cell cycle arrest
Cytochrome C
Apoptosis
Procaspase9
Apaf1
ATP
Apoptosome
PARP1
Procaspase3
Procaspase7
Cleaved PARP1
Caspase3
Caspase7

frequency of chromosome 11q deletions (Stilgenbauer S *et al*, 1997, Stoppa-Lyonnet D *et al*, 1998). As indicated above, T-PLL was also known to occur at a much higher frequency in individuals with Ataxia Telangiectasia (AT), and interestingly the T-PLL tumour cells from both AT and non-AT individuals had been shown to be characterised by the presence of recurrent chromosomal translocations involving the T-cell receptor genes. This had suggested that the acquired loss of ATM function, via sporadic *ATM* mutations, might be important in the development of T-PLL in non-AT individuals (Stilgenbauer S *et al*, 1997, Taylor AM *et al*, 1996).

In contrast to T-PLL, CLL was known to be a tumour that is characterised most commonly by chromosomal deletions rather than translocations (Dohner H *et al*, 2000). However, a distinctive feature of CLL tumour cells which had been described was their in vivo resistance to apoptosis (Binet JL *et al*, 1996). Since loss of functional ATM was known to impair the induction of apoptosis following DNA damage this provided another reason to support the belief that sporadic mutations in the *ATM* gene might occur in B-CLL, as well as in T-PLL tumours (Rotman G, Shiloh Y, 1998).

1.5.2 Nature and distribution of *ATM* mutations in lymphoid tumours

The majority of the mutations that occur in the germline of patients with ataxia telangiectasia are non-sense changes that lead to the generation of a premature stop codon and would be predicted to give rise to a truncated protein (Stankovic T *et al*, 1998). This protein is usually unstable and rapidly degraded and therefore patients that inherit bi-allelic truncating mutations have no detectable ATM protein in their cells and have absent ATM function

(Lakin ND, 1996). In contrast, the majority of mutations that have previously been identified in T-PLL, MCL and CLL tumours have been mis-sense changes that will give rise to an amino acid substitution in the translated protein (Stilgenbauer S *et al*, 1997, Stoppa-Lyonnet D *et al*, 1998, Stankovic T *et al*, 1999, Schaffner C *et al*, 1999, Bullrich F *et al*, 2000). The consequence of these changes on the function of the translated protein will depend on the location and nature of the substitution.

The *ATM* mutations that were previously detected in T-PLL had tended to be clustered within the more highly conserved C-terminal region of the *ATM* gene and these were invariably associated with loss of heterozygosity at the *ATM* locus (Stilgenbauer S et al; 1997, Stoppa-Lyonnet D et al; 1998). By comparison, the mis-sense changes that had previously been detected in CLL tumours were found to be distributed across the gene with no obvious clustering pattern (Stankovic T et al; 1999, Schaffner C et al; 1999, Stankovic T et al; 2002).

1.5.3 Functional effects of *ATM* mutations in CLL tumours

Certain aspects relating to the role of *ATM* mutations in the pathogenesis of CLL tumours have been previously addressed.

In line with the known defect in response to DNA damage in AT cells, CLL tumours with acquired bi-allelic abnormalities in the *ATM* gene have been shown to have a reduced ability to repair chromosomal breaks that are induced following irradiation (Stankovic T *et al*, 2002). Furthermore, compared to tumours with wild type *ATM*, CLL tumours with *ATM* mutations have been shown to fail to up regulate the level of the p53 protein after the induction of

DNA DBSs and to fail to induce the expression of the p53 regulated p21 protein. In addition, CLL tumours with *ATM* mutations also had a defect in the induction of apoptosis following DNA damage with irradiation and this apoptotic defect was noted to be less marked than the defect observed in CLL tumours with mutations in the *TP53* gene (Stankovic T *et al*, 2002, Pettitt A *et al*, 2001).

Gene expression profiling following DNA damage with irradiation has also been studied and this has confirmed that CLL tumours with *ATM* mutations fail to induce p53-dependent pro-apoptotic transcriptional responses. Interestingly, tumours with *ATM* mutations also failed to activate a number of p53-independent pro-survival transcriptional responses such as those mediated by NFkappaB (NFKB) and various Heat Shock Proteins (HSPs) (Stankovic T *et al*, 2004). This dual effect of ATM in affecting both pro-apoptotic responses and pro-survival responses would explain the observation that the apoptotic defect in CLL tumours with impaired ATM function is less marked than the defect observed in tumours with impaired p53 function.

One aspect of the response to DNA damage with irradiation that has not been previously studied in CLL tumours is the early ATM dependent phosphorylation of target substrates. Since ATM directly catalyses these reactions, they are likely to most reliably reflect the level of ATM function in the CLL tumour cells. Recently, there has been development of commercial antibodies that bind to proteins that are phosphorylated on specific residues and this has now made the assessment of these responses more straightforward to study (Kurz EU, Douglas P, Lees-Miller SP, 2004). The

identification of tumours with functional impairment of these phosphorylation responses might therefore provide an alternative means to detect CLL tumours that have defective ATM function as a result of the presence of *ATM* mutations.

1.5.4 Relationship between *ATM* mutations and prognostic features

The relationship of *ATM* mutations with other prognostic factors is a further area that has the potential to enhance our understanding of how *ATM* mutations might contribute to the development and progression of the CLL tumour clone.

1.5.4.1 Clinical features of CLL

The potential association between *ATM* mutations and clinical features of CLL such as age, gender, LDT and disease stage has not been specifically addressed. However, in one published study a high proportion of patients with Binet stage B and C had *ATM* mutations (Stankovic T *et al*, 2002). This might suggest that *ATM* mutations are associated with a more aggressive disease phenotype, but ideally the assessment of such a relationship requires that the cohort of CLL patients studied is representative of the whole spectrum of different CLL phenotypes seen in haematology clinics.

1.5.4.2 *IGVH* mutation status

One biological marker that has been investigated in relation to *ATM* mutations in CLL tumours is the mutation status of the *IGVH* genes. In a previous series of CLL tumours, *ATM* mutations were found to occur

exclusively in CLL tumours with un-mutated *IGVH* genes. However, in this study a high proportion of the total patient population had un-mutated *IGVH* genes and, therefore, the possibility remains that *ATM* mutations might occur in both *IGVH* subgroups (Stankovic T *et al*, 2002). An association of *ATM* mutations with un-mutated *IGVH* genes could indicate that *ATM* mutations are more likely to develop in those CLL tumours with unmutated *IGVH* genes but, alternatively, might suggest that the loss of ATM function is a factor in the transformation of process of a cell that has an impaired ability to undergo the process of somatic hyper-mutation (Stankovic T *et al*, 2002).

1.5.4.3 Deletions of chromosome 11q

There is only limited data regarding the relationship between CLL tumours with an 11q deletion and tumours with *ATM* mutations. All tumours with an 11q deletion, as detected by FISH, will have loss of one *ATM* allele and therefore if *ATM* behaves as a tumour suppressor gene one might expect a close association between the two genetic subgroups of CLL tumours (Schaffner C *et al*, 1999, Dewald *et al*, 2003). One research group performed mutation screening of the C-terminal region of the *ATM* gene in 22 CLL patients with an 11q deletion and found *ATM* mutations in just 5 cases (Schaffner C *et al*, 1999). Conversely, in the largest previous study reporting on *ATM* mutations in CLL tumours, loss of heterozygosity at the *ATM* gene loci was only demonstrated in 2 out of 16 patients (Stankovic T *et al*, 2002).

The cellular consequences of an 11q deletion on the DNA damage response in CLL tumours have not been studied and the role of abnormalities in ATM function in accounting for the poor clinical outcome in this group of

patients remains uncertain (Dohner H et al, 1999). Specifically, any potential differences in the integrity of the damage response pathway between those 11q-deleted CLL tumours with an additional *ATM* mutation and those 11q-deleted tumours with a second wild type *ATM* allele, which are thus heterozygous at the *ATM* locus, have not been investigated.

The cells from AT carriers, which are heterozygous at the *ATM* gene locus as a consequence of germ-line *ATM* mutations, have normal responses to DNA damage both in terms of the *in vitro* repair of chromosomal breaks and the activation of p53 dependent apoptosis. Furthermore, these carriers do not exhibit the phenotypic characteristics of AT patients (Taylor AM et al; 1975, Unpublished data). However, there are several lines of evidence that indicate that inherited heterozygous *ATM* germ-line mutations might indeed exert subtle cellular effects. For example, family and epidemiological studies have shown that carriers of mono-allelic germ-line *ATM* mutations have a small increased predisposition to cancer development (Thompson D *et al*, 2005, Thortenson Y *et al*, 2003, Dork T *et al*, 2001, Renwick A *et al*, 2006). Secondly, the presence of certain *ATM* heterozygous sequence variants in breast cancer patients has been linked to the development of post-radiotherapy fibrosis (Andreassen CN *et al*, 2006). Finally, a recent animal study has demonstrated that haplo-insufficiency of the *ATM* gene leads to a two to three fold enhanced susceptibility to carcinogen induced mammary tumours (Lu S *et al*, 2006).

1.5.4.4 Other genetic defects

The relationship between *ATM* mutations and other chromosomal abnormalities has not been specifically addressed but notably tumours harbouring *TP53* mutations, which commonly associate with 17p deletions, have been found to be generally distinct from tumours with *ATM* mutations. This suggests the possibility that the two abnormalities could be mutually exclusive in CLL tumours, although mutations in both genes have been reported in diffuse large B-cell lymphoma (DLBCL) (Gronbaek K et al; 2002).

1.5.5 The temporal acquisition of *ATM* mutations in CLL tumours

The timing of the development of *ATM* mutations in relation to the ontology of CLL can also provide a useful insight into their contribution to the pathogenesis of the disease.

1.5.5.1 Germ-line changes in the *ATM* gene

The possibility that *ATM* mutations could contribute to the genetic predisposition of CLL has been previously considered. One reason to support this suggestion is associated with the clinical phenotype of AT patients. These individuals, who have bi-allelic germ-line *ATM* mutations, have a markedly increased incidence of lymphoid tumours which include B-cell malignancies (Thompson D *et al*, 2005, Oscier D *et al*, 2004). Notably, however, there are no reports to date of CLL developing in an AT patient. However, it is possible that AT patients do not survive long enough to develop CLL, which is primarily a malignancy of middle and old age.

Recently, it was found that a minority of the *ATM* mutations, which have been detected in CLL patients' tumour cells, are also present in germ-line material of those CLL patients (Stankovic T *et al*, 2002). Therefore, in these individuals the CLL had arisen in *ATM* mutant heterozygous carriers. Furthermore, the frequency of the CLL patients that had these germ-line *ATM* mutations was higher than the previously estimated incidence of heterozygous *ATM* mutation carriers in the UK population (Stankovic T *et al*, 1998). This, therefore, suggested the possibility that these carriers might be at an increased risk for the development of CLL. Conversely however, a recent publication that assessed cancer risk in the relatives of AT patients did not find any increase in the incidence of CLL in heterozygote *ATM* mutation carriers and in fact no cases of CLL were identified in this population (Thompson D *et al*, 2005).

Initial studies on CLL family pedigrees did not find any conclusive evidence to support a role of *ATM* mutations in the development of familial CLL. For example, in one study, there was no evidence of linkage between the development of CLL and the presence of specific *ATM* polymorphic markers amongst different family pedigrees. A second study investigated *ATM* mutations in CLL family pedigrees. Although, several *ATM* mutations were identified in individual CLL patients, there was no evidence for the segregation of any of these *ATM* mutations amongst affected family members (Bevan S *et al*, 1999, Yuille M *et al*, 2002).

In contrast to these initial studies, the largest report on CLL pedigrees to date used a genome wide linkage-scan of single nucleotide polymorphisms (SNPs), and this study did find some evidence of linkage of chromosome 11q,

although this was not statistically significant (Sellick GS *et al*, 2005). In addition, a role for the *ATM* gene in the development of CLL has recently emerged from a large case-control study. This study found evidence linking certain *ATM* SNPs with CLL patients compared to controls. The *ATM* gene was one of only three genes from a genome-wide analysis that demonstrated such an association (Rudd et al; 2006).

Therefore, current data supports the concept that abnormalities in the *ATM* gene might have a role in the predisposition for the development of CLL but that if this is the case it is likely to be a low penetrance effect.

1.5.5.2 Acquired *ATM* mutations

The presence of germ-line *ATM* mutations suggests the possibility that they might have a role in the process of clonal transformation of CLL tumours (Stankovic et al; 2002). However, the majority of the *ATM* mutations that have been identified to date have been sporadic changes and in most of these cases the timing of their development with respect to clonal transformation was unknown (Stankovic T et al; 2002, Schaffner C et al; 1999, Stankovic T et al; 1999, Bullrich et al; 2000). Intriguingly, in one study, two of the *ATM* mutations that were detected in tumour cells were shown to be absent from the patient's germ-line but to be present in non-tumour cells including T-lymphocytes and monocytes. The pathogenic role of the mutations in the non-tumour cells remains obscure but this data provides evidence for the presence of *ATM* mutations at the time of clonal transformation suggesting a potential causative role (Stankovic T *et al,* 2002).

By comparison there is evidence to indicate that the development of other genetic defects, such as the loss of chromosomes 17p and 11q, often occurs some time after clonal transformation in CLL tumours. This is indicated by the fact that they are not always present in 100% of the tumour cell clone and has also been formally demonstrated by the analysis of serial samples from individual patients (Cuneo A *et al*, 2002, Dewald GW *et al*, 2003). By analogy, it is also therefore possible that *ATM* mutations could occur during disease progression at a time after the initial formation of the clone in certain tumours. However, the late acquisition of *ATM* mutations in CLL tumours has not been reported.

1.5.6 Consequences of *ATM* mutations on disease outcome

The consequences of *ATM* mutations on disease outcome in CLL patients have not been previously assessed. These will be important in the evaluation of *ATM* mutations as a prospective prognostic marker in CLL. Furthermore, measurement of their effects on clinical outcome will also provide clinical evidence to corroborate the previous *in vitro* studies, which suggest that *ATM* mutations influence the behaviour of the CLL clone.

The detrimental effect of *ATM* mutations on the induction of apoptosis following DNA damage with irradiation *in vitro* has been demonstrated (Stankovic T *et al*, 2002). This would indicate that there could be resistance to tumour killing *in vivo* following DNA damage in patients whose tumours had defective ATM function. Such damage might occur intrinsically within the tumour cells through the generation of oxygen radicals, but could also be the result of extrinsic damage induced by the use of DNA-damaging

chemotherapeutic drugs (Barzilai A, Yamamoto KI, 2004, Kurz EU, Lees-Miller SP, 2004). Thus, given the function of ATM, one might predict that *ATM* mutations would impair the outcome of CLL patients and that this impairment would be due in part to resistance to certain DNA damaging chemotherapeutic drugs. However this has not yet been demonstrated.

The majority of the previous in vitro work has focussed on the effect of *ATM* mutations on the responses to damage with irradiation, and the role of *ATM* mutations in the responses to the chemotherapeutic drugs used to treat CLL patients is largely unknown (Stankovic T *et al*, 2002, Pettitt AR *et al*, 2001, Stankovic T *et al*, 2004).

1.6 Mechanism of action of chemotherapeutic drugs in CLL tumours

The common first-line regimes used to treat CLL patients in the UK are chlorambucil or fludarabine mono-therapy, or the combination of fludarabine with cyclophosphamide.

1.6.1 Chlorambucil

Chlorambucil belongs to the class of drugs known as nitrogen mustards. It induces alkylation of CLL lymphocyte DNA, which includes the development of purine-drug complexes and also the formation of both intra-strand and inter-strand cross links (ICLs) at N-7 guanines. The DNA ICLs are considered to be the most important for the cytotoxicity of chlorambucil and the cellular processing of these ICLs is believed to result in the generation of DNA DSBs. Furthermore, there is evidence that these breaks are subsequently repaired by non-homologous end joining proteins in a similar manner to the DSBs

generated by IR in CLL cells. It is therefore not unexpected that there is cross-resistance between ionising irradiation and chlorambucil in CLL tumours. However the specific role of ATM in the mechanism underlying this resistance to chlorambucil is not known (Panasci L *et al*, 2001).

1.6.2 Fludarabine

Fludarabine (9-beta-D-arabinosyl-2-fluoroadenine-5'-monophosphate) is a purine analogue and it is converted within the cell into its 5'-triphosphate derivative. Phosphorylated purine analogues primarily induce cytotoxicity due to their inhibition of DNA synthesis. This occurs due to a direct inhibitory effect on ribonucleotide reductase but also as a result of the incorporation of purine analogues into replicating DNA. Once incorporated into DNA there is cessation of further DNA strand elongation and if the cell fails to repair this lesion it will ultimately result in the activation of apoptosis (Pettitt AR; 2003). For fludarabine, there is evidence that its incorporation into DNA leads to the activation of p53 and also to the generation of cleaved caspase 3 and PARP1 (Achanta G *et al*, 2001, Rosenwald A *et al*, 2004, Rao VA, Plunkett W, 2001).

Interestingly, the majority of circulating CLL cells is non-cycling and arrested in G1 and therefore there is no active DNA replication occurring in these cells. Therefore, it has been suggested that purine analogues become incorporated into the DNA in these cells during the repair process following DNA damage. This DNA damage might occur as a result of intrinsic cellular process such as the generation of oxygen radicals, or alternatively might be the result of damage induced by other chemotherapeutic drugs (Pettitt AR, 2003). The combination of fludarabine with the alkylating agent

cyclophosphamide results in a more than additive apoptotic cell death compared to the sum of the effects of either drug alone, and, this has been shown to be the result of fludarabine inhibiting the repair of the lesions induced by cyclophosphamide (Yamauchi T *et al*, 2001). These observations provided the rationale for the using the combination of these drugs for treating CLL patients

The role of ATM in the response pathway to nucleotide analogue-containing DNA has not been investigated. Interestingly, however, there is one study implicating the related PIKK repair protein, DNA-PK, in this response (Achanta G *et al*, 2001). Furthermore, there is also one report showing that CLL tumours with *ATM* mutations demonstrate increased resistance to apoptosis following treatment with fludarabine. However, in this study, the potential mechanism of action to account for this finding was not addressed (Alvi A *et al,* 2005). It also remains unclear whether fludarabine incorporation leads to the formation of DNA DSBs and, if these do occur, whether there is activation of ATM dependent phosphorylation responses, in a manner analogous to the response to the DNA DSBs induced by irradiation.

Aims

The determination of disease factors that have prognostic value has led to an increase in the understanding of the nature of CLL and this has had implications in the clinical management of patients.

ATM mutations have been previously identified in CLL tumours but their consequences on the outcome of CLL patients and their potential role as a prognostic marker has not been evaluated. In this project, therefore, I aimed to study a large cohort of CLL patients in order to address the prevalence of *ATM* mutations and their effects on clinical parameters such as the overall survival and treatment free survival from diagnosis. In addition my aim has been to investigate the relationship between *ATM* mutations and other prognostic features in CLL, in order to determine if *ATM* mutations can provide independent prognostic information.

An important focus was to study the relationship between CLL tumours with *ATM* mutations and those with deletions of chromosome 11q, and hence loss of one *ATM* allele. Therefore, it was my intention to address the subsets of CLL tumours that have a mono-allelic *ATM* defect, due to either a deletion or a mutation, and the subsets that have bi-allelic defects, due to combinations of mutations and deletions. The principal function of the ATM protein is in the coordination of the cellular response to DNA damage in the form of DSBs. In this project, I wanted to investigate the consequences of mono-allelic or bi-allelic *ATM* defects on the integrity of this DNA damage response pathway, and specifically I have aimed to study whether the presence of *ATM* mutations can stratify CLL tumours with an 11q deletion into

two subgroups in terms of their cellular phenotypes but also the clinical outcome of CLL patients.

Previous studies have concentrated on the effects of *ATM* mutations on the consequences of DNA damage that is induced by irradiation. In this project, my aim was to study the role of ATM in the response to the chemotherapeutic drug, fludarabine, which is used as a first line therapy in CLL patients. Importantly, I was interested to understand whether tumours with *ATM* mutations have impairment in the response to fludarabine and whether this may explain the resistance of a subset of CLL tumours to this chemotherapeutic drug.

The timing of the development of *ATM* mutations is important in their evaluation as a prognostic marker and in addition provides an important insight into the role of ATM in the pathogenesis of CLL tumours. I have therefore aimed to study whether the *ATM* mutations detected in CLL tumours represented germ-line changes or acquired changes. I also wished to compare the frequency of germ-line *ATM* gene changes between the CLL patients and a control population in order to address whether germ-line *ATM* mutations can predispose to the development of CLL. In the case of the acquired *ATM* mutations, I wanted to investigate whether they were present at the time of diagnosis or developed some time during the CLL tumour progression to gain further insight on how they might impact on the behaviour of the tumour clone.

Materials and Methods

3.1 Samples from CLL patients and controls

The diagnosis of B-CLL was confirmed in each case by immunophenotyping. Specifically, this required the demonstration of a clonal population of B-lymphocytes with either Kappa or Lambda light chain restriction, together with the co-expression of surface CD5 with CD19 and CD23, in association with low density surface immunoglobulin expression. The studies were performed according to local ethical guidelines and written informed consent was obtained from all patients.

Two cohorts of CLL patients were studied. The first consisted of 155 CLL patients and included consecutive patients that attended the haematology outpatient clinics at Selly Oak and Heartlands Hospitals in Birmingham over the 14-month period from December 2001 to January 2003. On the day of their clinic visit, fourteen millilitres of peripheral blood was collected from each patient into heparinised tubes.

The second cohort of 53 CLL patients were all characterised by the fact that they had previously been found to have a deletion of chromosome 11q in their tumour cells by FISH analysis. The patients originated from Birmingham, Bournemouth and Leicester hospitals. This FISH analysis had been performed in regional genetics units for clinical indications or as part of independent studies. In many of these cases the processing of blood samples and the extraction of DNA had been performed at local hospitals.

The control cohort consisted of 71 Caucasian healthy volunteers over 65 years in age and 90 anonymous individuals who were blood donors. Fourteen millimetres of whole blood was collected from the 71 volunteers. The DNAs from the blood donors formed part of a pre-existing bank that was made available for this study.

3.1.1.Separation of Mononuclear Cells from CLL blood samples

Whole blood samples were diluted with RPMI in a 1:1 ratio in universal tubes and layered on to 10mls of lymphoprep (Nycomed). Samples were centrifuged at 1600 rpm for 20 minutes. Mononuclear cells were retrieved from the interface and washed twice with RPMI. Cells were counted and stored at concentrations of up to 5 x 10^7cells/ml in 90% fetal calf serum (FCS) and 10% Dimethyl Sulphoxide (DMSO) in 1ml vials. Vials of cells were initially stored in insulated boxes at -80ºC and once frozen were transferred to liquid nitrogen.

3.1.2. Germ-line Material

Buccal cells were obtained from the CLL patients using a mouth swab. Cells were washed in phosphate-buffered saline (PBS) and stored as a pellet at -20ºC.

3.1.3 Diagnostic Material

On selected samples, archive tumour material cells was available in the form of DNA, or cells that had been collected at the time of diagnosis of a patient's CLL.

3.1.4 DNA extraction

Genomic DNA was extracted from frozen tumour cells using the QIAamp DNA blood minikit (QIAGEN, Crawley, West Sussex, UK). Samples were thawed and cells were washed with RPMI culture medium. 5 x 10^6 cells were diluted in 200µl of phosphate-buffered saline (PBS) and incubated with 200µM lysis buffer and 20µM protease at 56ºC for ten minutes. 200µl of 100% (v/v) ethanol was added in order to precipitate the DNA. The mixture was then added to the QIAamp spin column in a 2ml collection tube and micro-centrifuged at 8000rpm for 1 minute. The column was transferred to a new collection tube and washed initially with buffer containing ethanol provided with the kit. The column was placed in a 1.5ml micro-centrifuge tube and 200µl of elution buffer was added to the DNA and incubated for 5 minutes, followed by micro-centrifugation at 8000rpm for 1 minute. Subsequently, samples were diluted with elution buffer to give a final concentration of DNA of approximately 50ng/µl.

DNA was also extracted from the control blood samples. Initially, the whole blood samples were spun at 1600rpm for 20 minutes to allow separation into plasma, buffy coat and red cell compartments. 200µl of blood from the buffy coat layer was then directly incubated with 200µM of the lysis buffer and 20µM of protease. The subsequent extraction procedure was similar to that described above and the final DNA was eluted into a volume of 200µl, with no further dilution steps required.

Extraction of DNA from the buccal cells was performed by the same method but the DNA was eluted into 30µl of elution buffer.

3.2. Screening for *ATM* and *TP53* mutations

3.2.1 Polymerase Chain Reaction (PCR) for the *ATM* and *TP53* genes

I performed separate PCR reactions for each of the 62 *ATM* coding exons (4-65) using 60 primer pairs (Table 3.1). Exons 4 and 5, and, exon 43 and 44 were each expanded using a single PCR reaction using primers located outside both exon coding sequences, as these pairs of exons are positioned close together within the gene sequence. The PCR reactions for each exon were performed in 96-well plates on up to 96 tumour samples at a time. Each reaction used 50ng of gDNA, 0.2μM of dNTPs (Roche), 0.2μM of each primer (Sigma), 1.5mM of MgSO4 and 1 unit of the high stringency DNA polymerase, Optimase (Transgenomic). These were mixed with sterile water to form a total reaction volume of 50μl. PCR conditions were 5 minutes at 95˚C, followed by 40 cycles of 30 seconds at 95˚C for denaturation of dsDNA, 30 seconds at 53˚-58˚C for primer annealing and 1 minute at 72˚C for polymerase dependent DNA extension, and final extension of 7 minutes at 72˚C. Primer annealing temperatures were predicted using the 'Mutation Discovery' software programme (Transgenomic) (Table 3.1).

TP53 exons 3 to 10 were amplified using 5 primer pairs (AltaBiosciences, University of Birmingham). These coding exons were chosen because they have been shown to harbour the majority of the pathogenic mutations that occur in the *TP53* gene (Vousden KH, Lu X, 2002). Exons 3 and 4, exons 5 and 6, and exons 8 and 9 were each amplified in a single PCR reaction using

Table 3.1 – PCR and DHPLC conditions for exons 4-65 of *ATM* gene

Exon	Primer Sequences	PCR annealing temp (ºC)	DHPLC Temp (ºC)
4/5	Forward – CACACCTCTTTCTCTCTATATATGC Reverse-GGAAGCAAAGATAAATGTTAAGACTTACAC	59	53.5
6	Forward-ATTGTTCTTGTAGGAGTTAGGCCTTG Reverse-AAAAACTCACGCGACAGTAATCTG	58	54.0
7	Forward-TAAATAGTTGCCATTCCAAGTGTC Reverse-TGGTGAAGTTTCATTTCATGAGG	58	54.7
8	Forward-CCTTTTTCTGTATGGGATTATGGA Reverse-TACTGAGTCTAAAACATGGTCTTGC	58	55.5
9	Forward-TTCTTTCAGCATACCACTTCATAAC Reverse-TGAATGAAGAAGCAAATTCAAAACAG	58	55.0
10	Forward-TGGGAGCTAGCAGTGTAAACAGAG Reverse-CAGGAAATTTCTAAATGTGACATGAC	58	54.6
11	Forward-GCTCAAAAAAAAAAAAAAGAAAAAAGTGG Reverse-AAATGACTTAGTTCTGGTTGAGATG	56	54.7
12	Forward-TCCTTTAGTTTGTTAATGTGATGG Reverse-ACTATGAAAATGATCAGGGATATG	56	56.2
13	Forward-CCTCCAATAGCTTGCTTTTCAC Reverse-AAACAGCAGCATGCTAATGAAC	58	55.8
14	Forward-GTATTCTTTACATGGCTTTTGGTC Reverse-TACTACCCAGCTAAAATTATCATC	56	55.0
15	Forward-CATATAAGGCAAAGCATTAGGT Reverse-CCTATTTCTCCTTCCTAACAGT	56	55.5
16	Forward-GAATTTGTTCTTACAAAAGATAGAG Reverse-GAATACATTTCATTCAAATTTATCCGA	55	55.5
17	Forward-GTATGTCCAAGATCAAAGTACACTG Reverse-GGGTGACAGAGAAAGATCCTATC	58	55.5
18	Forward-GTTTTTATTTCTTTGTTGCTTGGTTCT Reverse-CAAATATGATAGCAAAACAGGAAGC	58	55.9
19	Forward-CTCCCAAATTGCTGAGATTACAGATG Reverse-ATGAGGCCTCTTATACTGCCAAATC	58	56.4
20	Forward-ATATATGGCTGTTGTGCCCTTCTC Reverse-CATCAGATAAAATCCAAGAGCTTC	58	56.5
21	Forward-AAACCTGATTTTTTTCCCTCCTAC Reverse-TTTATAAGCTTAACAGAACACATCAGT	58	54.4
22	Forward-AATAACTGATGTGTTCTGTTAAGC Reverse-AAACTTGCATTCGTATCCACAGAT	56	55.9
23	Forward-GTAACTTATTAATAACCTTTAAGTGAG Reverse-ACTCATTAACAAAACAAAGACTGCT	56	54.6
24	Forward-CTATTTCATATTTAACCACAGTTC Reverse-TATGTAAGACATTCTACTGCCATC	56	56.7
25	Forward-TTGTTTGTTTGTTTGCTTGCTTGTTT Reverse-CATATGATAACAGCAAATACATGTTAC	56	57.4
26	Forward-GTCAAAAAATCTGGAGTTCAGTTG Reverse-GGAAGCTTCTAATAAAATACTCATC	56	55.0
27	Forward-GAATGTTGTTTCTAGGTCCTACTC Reverse-GTGAGGGGGACTTGCTAAGTATTG	58	54.0
28	Forward-CTTGGAAAAGTTATATATAACCTG Reverse-AACTTAAAGGTTATATCTCATATC	53	55.0
29	Forward-TTTGAGCTGTCTTGACGTTCACAG Reverse-TTGAAATAGACATTGAAGGTGTCAAC	58	55.5
30	Forward-TTTTCATTTTGGAAGTTCACTGGTC	56	55.0

	Reverse-GGAATGTTCTATTATTAAACTCATC		
31	Forward-GTGTATTTATTGTAGCCGAGTATC Reverse-GGAAGAACAGGATAGAAAGACTGC	56	54.5
32	Forward-GACTTGTGAATGAATTTATTTCAGAG Reverse-CACTCAAATCCTTCTAACAATAC	56	56.5
33	Forward-TTACAGTAAGTTTTGTTGTCTTAC Reverse-CAGATTTTTGAAAAGTACTACTATG	56	54.0
34	Forward-CCAATACGTGTTAAAAGCAAGTTAC Reverse-AACAGGTAGAAATAGCCCATGTC	58	57.4
35	Forward-AAACAAAAGTGTTGTCTTCATGCT Reverse-TCCTATATGTGATCCGCAGTTGAC	58	57.2
36	Forward-GTTTTATGTATGATCTCTTACCTATG Reverse-GAAGTATCATTCTCCATGAATGTC	58	55.5
37	Forward-CAGTCCACCTTAACATTCATCAAG Reverse-ACAGTCATGACCCACAGCAAACAG	58	55.6
38	Forward-AAGGTACAATGATTTCCACTTCTC Reverse-CAGCACTCTTTAGATAAACAGGTC	58	56.0
39	Forward-AGCAGTATGTTGAGTTTATGGCAG Reverse-GGATTCCATCTTAAATCCATCTTTC	58	55.2
40	Forward-CCTTATAGCATAGTGGGAGACAG Reverse-CAAGTTACACTCTAGATCCTAAACG	58	56.0
41	Forward-TAAGCAGTCACTACCATTGTATTC Reverse-TACCCTTATTGAGACAATGCCAAC	58	54.3
42	Forward-CAGGAGCTTCCAAATAGTATGTTC Reverse-CACATGGCATCTGTACAGTGTCT	58	54.5
43/44	Forward-TAGAGTTGGGAGTTACATATTGGT Reverse-GCACTACACTAGTGATGGCTTTAC	58	55.4
45	Forward-GTTTTTCTGTTGATATCTTTGATTAC Reverse-GAGAGGCAAAAAAAAAAAAATCAAGTC	56	55.7
46	Forward-GTCCTTTGGTGAAGCTATTTATAC Reverse-CTCAAGTTTTCAGAAAAGAAGCCA	58	54.7
47	Forward-CCTCTTCTTTATTTTCAGAGTGTC Reverse-GTCACTATTGGTAACAGAAAAGC	58	58.0
48	Forward-CATTTCTCTTTGCTTACATGAACTC Reverse-TAGAGATCTCTATCTCTTAATGAC	53	57.0
49	Forward-CATGGTAGTAGTATCAGTAGTAAAAG Reverse-CAGTAAAACACTAATCCAGCCAAT	58	57.5
50	Forward-AGTTGGGTACAGTCATGGTAATGC Reverse-CTAAGTAACTATCTTAAGGGTTGCTC	58	57
51	Forward-GTGTATTACCTTAATTTGAGTGATTC Reverse-AAGACCAAGTCACTCTTTCTATGC	58	56.1
52	Forward-ATCATGTGTGATTTTGTAGTTCTG Reverse-TTCAAGCACAGGGTAGAATATTGG	56	56.8
53	Forward-TTGTGCTAATAGAGGAGCACTGTC Reverse-GTATTTCCATTTCTTAGAGGGAATG	58	54.4
54	Forward-TCTACCCACTGCAGTATCTAGAC Reverse-CAGCCTTGAACCGATTTTAGATG	56	55.3
55	Forward-TGTTGGGTAGTTCCTTATGTAATG Reverse-GGATTACGTTTGTGATTTTAAGCAG	56	55.6
56	Forward-ACTATTCCTGCTTGACCTTCAATG Reverse-GCCAATATTTAACCAATTTTGACC	56	54.7
57	Forward-TAAGTGCAAATAGTGTATCTGACC Reverse-CATCACTAAAACTCTAAGGGCTAAG	56	56.3
58	Forward-TTGCTATTCTCAGATGACTCTGTG Reverse-GCCTCCCAAAGCATTATGAATATG	56	55.9
59	Forward -CATCAAATGCTCTTTAATGGCCTT Reverse-TGCCAAACAAACAAAGTGCTCAATC	58	57.3
60	Forward-CATCTTTATTGCCCCTATATCTGTC Reverse-TGCCAAACAACAAAGTGCTCAATC	58	55.0

61	Forward-AAGAGATGGAATCAGTGATTTCAG Reverse-AGGCAAACAACATTCCATGATGAC	58	54.3
62	Forward-TTAGCTGTCAAACCTCCTAACTTC Reverse-TTGAGTAGCGGGATTACAGGTG	58	57.2
63	Forward-AGATATGTTGACAACATTGGTGTG Reverse-GAGATACACAGTCTACCTGGTAAG	58	55.0
64	Forward-GATACTGGTTCTACTGTTTCTAAG Reverse-AAAGGTTTCAGTGAGGTGAACAG	58	56.3
65	Forward-GGTGAGCAGTATTTTAAGAAGGTC Reverse-TCCCTACTTAAAGTAGTTGGCAG	58	56.8

primers designed to bind to sequences located outside the respective pairs of exons. PCR conditions were similar to those described above for the exons of the *ATM* gene, however primer annealing temperatures used for the *TP53* coding exons ranged from 58-63˚C (Table 3.2).

3.2.2 Denaturing High Performance Liquid Chromatography (DHPLC)

PCR products were screened for sequence changes by denaturing high performance liquid chromatography (DHPLC) using the WAVE® DNA fragment analysis system (Transgenomic). DHPLC detects sequence changes on the basis of the differential binding characteristics of heteroduplexes, which form when there is a sequence mismatch between two alleles, and homoduplexes, which form when two alleles are perfectly matched.

3.2.2.1 Heteroduplex formation

Prior to DHPLC analysis on the WAVE machine, each PCR product was processed in order to maximise the formation of any potential heteroduplexes. Samples were heated to 95˚C for 5 minutes to denature the double stranded DNA (dsDNA) into single stranded DNA (ssDNA). Samples were then cooled slowly by the decreasing the temperature by 1˚C per minute, from 95˚C to 65˚C using the PCR machine (Figure 3.1).

Certain CLL tumours were known to carry a chromosome 11q deletion and therefore would only have one *ATM* allele present. Therefore, a potential sequence change in the remaining allele might not be detected by DHPLC, as there would be no wild type allele with which to form a heteroduplex.

Table 3.2 – PCR and DHPLC conditions for exons 3-10 of *TP53* gene

Exon	Primer Sequence	DHPLC Temp (ºC)	PCR annealing Temp (ºC)
3 and 4	Forward-CCTAGCAGAGACCTGTGGGAAGCG Reverse-GCATTGAAGTCTCATGGAAGCCAGC	60	63
5 and 6	Forward-CTTTATCTGTTCACTTGTGCCCTGAC Reverse-GGGCCACTGACAACCACCCTTAAC	60	63
7	Forward-CTCCCCAAGGCGCACTGGCCTCATC Reverse-CAGGCCAGTGTGCAGGGTGGCAAG	58	60
8 and 9	Forward-CTGATTTCCTTACTGCCTCTTGCTTC Reverse-CTGGAAACTTTCCACTTGATAAGAGGTC	60	63
10	Forward-CTCAGGTACTGTGTATATACTTACTTC Reverse-GAATGGAATCCTATGGCTTTCCAAC	58	59

Figure 3.1

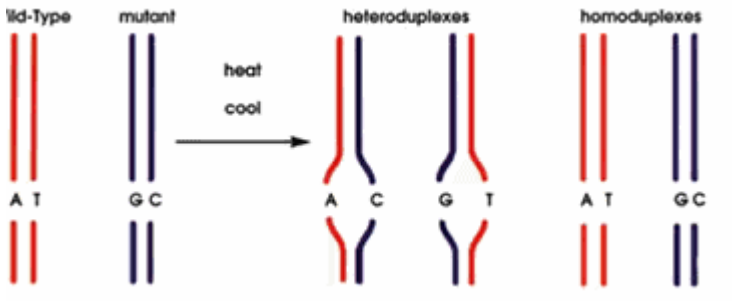
a. If there is a sequence change in one allele of a gene then a mixture of heteroduplexes and homoduplexes will form following denaturation and re-annealing.

b. The melting characteristics of exon 55 of the *ATM* gene are shown. Parts of the exon will be more completely denatured into single stranded DNA at a given temperature depending on the G-C content. At 53.5ºC, the whole of the coding region of the PCR product consists of dsDNA. At 55.5ºC, part of the exon remains as dsDNA, but part has denatured into ssDNA. Typically heteroduplexes are estimated to denature at a temperature 1ºC lower than homoduplexes. Therefore, at 55.5ºC, it might not be possible to detect all sequence changes because part of the exon had already completed denatured in the homoduplex. The ideal temperature to analyse this exon by chromatography would be thus be approximately 54.5 ºC.

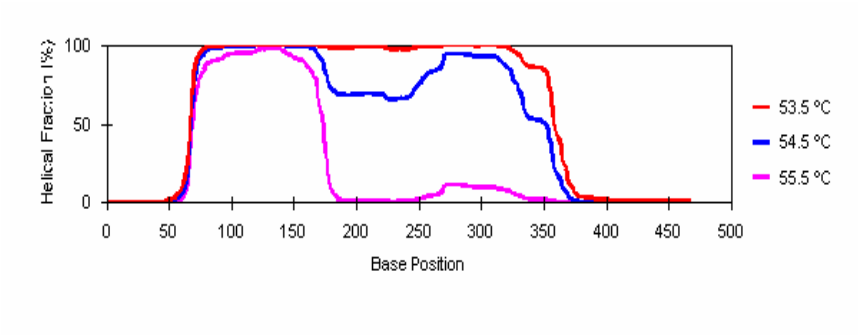
c. Heteroduplexes can be resolved from homoduplexes by chromatography because they will be more denatured over a given temperature range and will therefore elute earlier from a DNA binding column. In this example it would be possible to detect the presence of heteroduplexes over the range of temperature from 53 - 58 ºC.

Figure 3.1

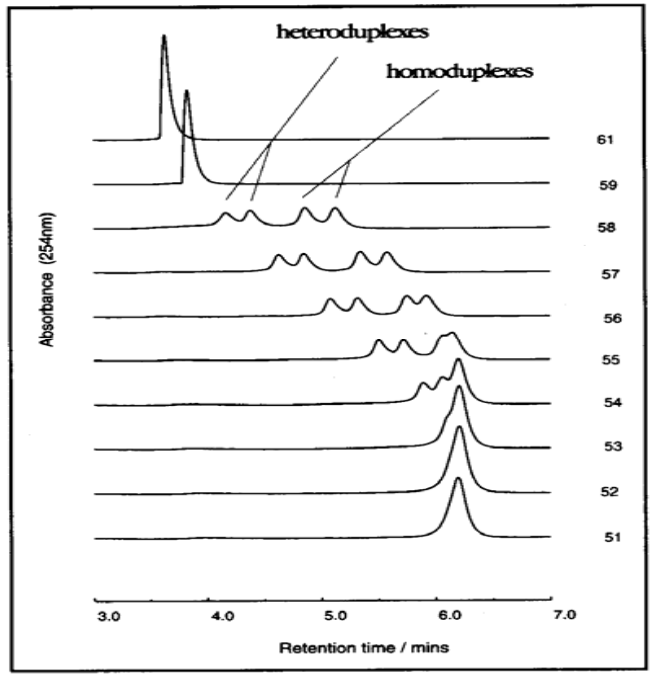
a



b



c



Therefore for these samples, 10µl of the final PCR product for each exon was mixed with 10µl of the product from a sample that was known to have both *ATM* alleles present, prior to the heteroduplex reaction. Subsequently both the mixed and unmixed products were analysed by DHPLC (Xiao W, Oefner PJ, 2001).

3.2.2.2 DHPLC

Heteroduplexes and homoduplexes have different properties that are exploited by DHPLC to screen for potential mutations. Due to the presence of a mismatch, heteroduplexes will denature into ssDNA over a lower temperature range than perfectly matched homoduplexes. Therefore, for a given temperature the heteroduplexes will be more completely denatured than homoduplexes. The WAVE machine (Transgenomic) has a DNASep column that contains hydrophobic divinylbenzene beads that bind DNA. The strength of the binding of DNA to the beads in the column will vary according to both the length of the DNA but also according to the degree of denaturation of the DNA.

During the process of chromatography the proportions of 2 buffers (Buffer A (0.1M Triethylammonium actetate (TEAA)) and Buffer B (0.1M TEAA and 25% acetonitrile) (Transgenomic)) are programmed to alter. Increasing the concentration of this Buffer B causes any DNA bound to the column to elute. However, due to the differential denaturation properties of heteroduplexes and homoduplexes, heteroduplexes will elute earlier than homoduplexes over certain temperature ranges. Therefore in order to detect sequence changes, the chromatography is performed at a programmed temperature that is

selected by the operator. This is chosen based on the denaturing characteristics of each exon. The ideal temperature will be one where the degree of denaturation is predicted to be the greatest between a homoduplex and a heteroduplex. This can be identified using the Wavemaker software (Figure 3.1). The choice of temperature is not always straightforward due to variations in the denaturing characteristics across any given exon. However, there is typically a range of temperatures over which it is theoretically possible to resolve the heteroduplexes from the homoduplexes. The temperatures for the DHPLC for each *ATM* exon are shown in Table 3.1.

The presence of the DNA that is eluted from the column is detected using a UV lamp and converted to a read-out on the screen, termed the chromatogram. Peaks can be seen that correspond to the heteroduplexes and homoduplexes. A single peak pattern is present if there are only homoduplexes present in the sample. Any alteration in this single peak pattern is indicative of the presence of heteroduplexes in the sample (Figure 3.2).

In this study, all chromatograms were analysed visually and chromatograms were compared across large numbers of samples for each exon. The PCR product was subsequently sequenced for all cases where a variant pattern demonstrated was detected.

3.2.3 DNA Sequencing

3.2.3.1 Purification and quantification

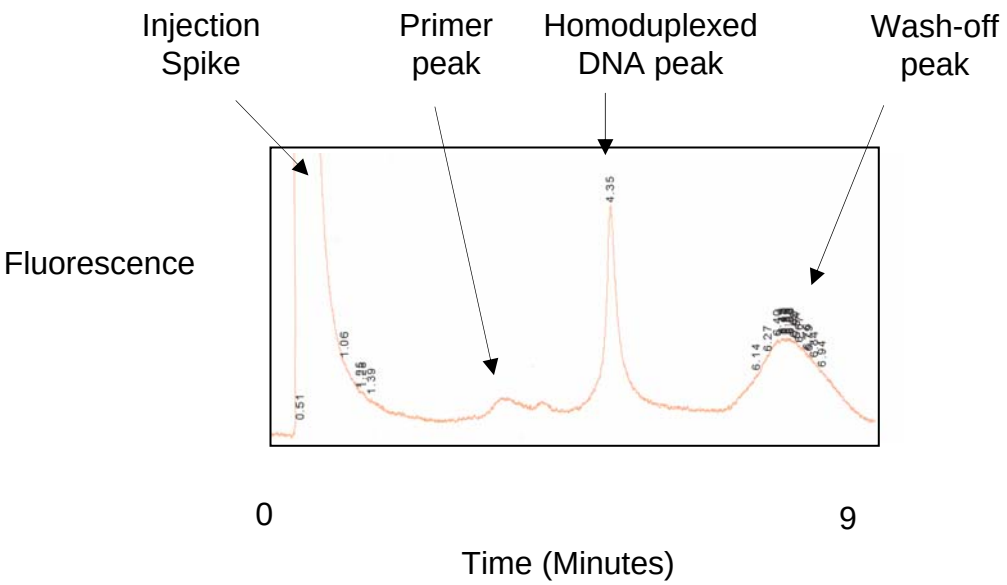
Prior to sequencing, all PCR products were purified using High Pure purification kit (Roche). Briefly, 40µl of each PCR product was mixed with

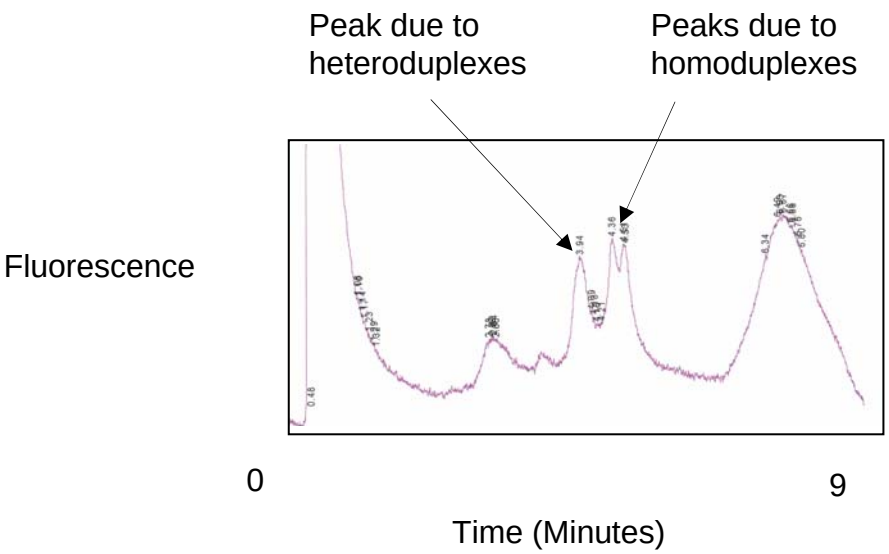
Figure 3.2

An example of a chromatogram is demonstrated for exon 10 of the *ATM* gene. In the absence of a sequence change a single peak pattern is seen that corresponds to the presence of perfectly matched homoduplexes. The injection spike, primer peak and the wash-off peak are also demonstrated.

By comparison in the presence of a sequence change an alteration in this single peak pattern is seen due to the presence of heteroduplexes and homoduplexes. In this example a triple peak pattern is seen.

Figure 3.2


Injection Spike
Primer peak
Homoduplexed DNA peak
Wash-off peak
Fluorescence
0
9
Time (Minutes)


Peak due to heteroduplexes
Peaks due to homoduplexes
Fluorescence
0
9
Time (Minutes)

200µl of binding buffer and spun at 14,000 rpm in a microcentrifuge. Products were washed twice with ethanol and the purified DNAs were eluted into 35 µl of the elution buffer.

The purified products were quantified visually by electrophoresing on an agarose gel. The agarose gel mix was made to a final concentration of 1.0% (w/v) in 0.5 x TBE containing ethidium bromides. 3µl of the PCR products and molecular weight marker were mixed with 2µl of Bromophenol loading dye, containing 0.25% (w/v) bromophenol and 40% (w/v) sucrose in SDW, and run at 80V for approximately 20 minutes. The gel was visualised by a UV transilluminator and recorded using Polaroid film (Genetic Research).

3.2.3.2 Labelling and precipitation of products

Sequencing was performed using the 3100 capillary sequencer from PE Applied Biosystems.

The labelling reactions were set up using 2µl Big Dye^{TM}Terminator v3.1 Cycle sequencing kit (PE Applied Biosystems), 6µl of 2.5Xsequence buffer (200mM Tris HCl, 5mM $MgCl_2$ pH9.0), 1µl primer (0.1µg/µl) and between 1-7µl of DNA (30-50ng) plus sterile water to make a total reaction volume of 20µl. Separate reactions were set up using forward and reverse primers for each exon. The labelling PCR was carried out for 25 cycles at 96˚C for 10 seconds, 50˚C for 5 seconds and 64˚C for 4 minutes.

The labelled products were precipitated with a 62.5µl of 100% (v/v) ethanol and 3µl 3M sodium acetate (pH 4.6) in 0.75ml tubes at room temperature for 15 minutes. The samples were then microcentrifuged at 14,000 rpm for 20minutes. The supernatant was removed and pellets were washed with 250

µl 75% (v/v) ethanol, followed by microcentrifugation at 14,000 rpm for 5 minutes. The supernatant was removed and the pellets were dried for 20 minutes.

3.2.3.3 The 3100 ABI prism™ DNA capillary sequencer

Prior to loading in the capillary sequencer, 10µl of 'High Dye™' was added to the pellet. Samples were denatured by heating to 100˚C for 5 minutes and put on ice for 2 minutes before being left at room temperature ready for use. The buffer chambers were first filled with 2.5x EDTA buffer (PE Applied Biosystems) and the water chambers filled accordingly. 3100 POP 6™ Performance Optimized Polymer was used to fill the 50cm sequencer capillary array (PE Applied Biosystems). The 3100 data collection software version 1.0 was set for sequencing at: Dye Set, E; Mobility file, DT3100POP6(BD)v2.mob; BIOLIMS Project, 3100_Project 1; Rum Module1, StdSeq50_POP6Default Module and Analysis Module 1, BC-3100_SeqOff.saz. The samples were loaded into the appropriate 96 well plates and run for approximately 2.5 hours. Following the run, the extracted samples were analysed using the ABI sequencing analysis version 3.6.1.

3.3 Protein chemistry techniques

3.3.1 Tissue Culture

Cells were thawed, washed once with culture media (RPMI with 10% FCS plus 1.2% penicillin and streptomycin, 1.2% L-Glutamine), re-suspended and

cultured in 24 well plates. Cells were cultured in incubators at 37ºC with 5% CO_2 and humidified air.

3.3.2 Tumour cell viability

Prior to further biochemical analysis the viability of the cells was assessed in each case. 10µl of suspended cells were mixed with 10µl of trypan blue and the cells were then visualised with a light microscope. Cells stained with trypan blue (dark) were considered as non-viable. Further biochemical experiments were only performed on samples where the baseline viability was demonstrated to be >80%.

3.3.3 DNA damage with irradiation

For experiments, involving the assessment of the response to DNA damage, each tumour sample was divided into aliquots according to the number of time points that were to be studied. Aliquots of cells were placed in up to 1 ml of culture media in universal containers and irradiated at a dose of 5 Gray using a Precisa 217 with source-emitting gamma-type rays (Pantatron, Gosport, Hampshire, UK). Following irradiation, cells were returned to the incubator and aliquots of cells were removed and processed at the appropriate time points.

3.3.4 Treatment with fludarabine

For experiments involving the investigation of the responses to fludarabine, tumour cells were cultured at a concentration of $1.25x10^6$ cells per ml of media. 2mls of re-suspended cells was aliquoted into each well of a 24-well

plate. Fludarabine (Schering) was re-suspended and diluted in culture media and added to the culture wells to give a concentration of 10 -20μM.

3.3.5 Protein extraction

Protein extraction from CLL samples was performed at defined time points according to individual experiments. At the appropriate time point, samples were transferred to universal containers and put on ice.

Two techniques were used for protein extraction in this project. In initial experiments, exactly $2x10^6$ cells were used for each time point. Cells were centrifuged at 1400 rpm for 5 minutes at 4ºC and the supernatant was removed, without washing in order to minimise any loss. Protein was extracted using a modified RIPA buffer containing 25mM Tris HCL (ph 7.6), 5mM disodium EDTA, 50mM sodium chloride, 30mM sodium pyrophosphate, 50mM sodium fluoride, and 1mM sodium orthovanadate plus 0.1 ml of 1% NP40 (Calbiochem), and 5μg of aprotinin and 10μg of Phenyl Methane Sulfonyl Fluoride (PMSF) in a volume of 10mls. 15μl of buffer was added to each pellet of cells and samples were thoroughly mixed by pipetting and transferred to 1.5ml microcentrifuge tubes. Samples were left on ice for 30 minutes and then spun at 12,000 rpm for 15 minutes in a microcentrifuge at 4ºC. The supernatant consisting of the protein lysate was removed and transferred to new 1.5ml tubes that had been previously placed on ice. Lysates were snap frozen in liquid nitrogen and subsequently stored until required at -80ºC.

In later experiments, cell pellets were washed once with cold phospho-buffered saline (PBS) and re-pelleted prior to protein extraction. Protein was

extracted using a TGN buffer (pH 7.5) consisting of 5ml 1M Tris (ph 7.5)(50mM), 5 ml 1M glycerophosphate (50mM), 6 ml 2.5M sodium chloride (150mM), 10 ml 10 % glycerol (BDH Laboratories), 1 ml 1% Tween 20, and 200µl NP40, together with water to form a total volume of 100mls. At the time of protein extraction the following protease and phosphotase inhibitors were added to 14.5mls of TGN buffer, namely 15µl Dithiothreitol (DTT) (1M), 150µl sodium orthanoate (0.1M), 300µl sodium fluoride (0.5M), 15µl 4-(2-Aminoethyl) Benzenesulfonyl Fluoridehydrochloride (AEBSF) (0.1M), 15µl Aprotinin (10mg/ml), 7.5µl Leucopeptin (10mg/ml) and 15 µl Pepstatin A (2mg/ml). Between 30 and 50µl of cold TGN buffer was added to each cellular pellet and mixed by pipetting. Lysates were subsequently obtained as described above.

The quantity of protein in the lysate was determined for all experiments that had not used fixed numbers of cells. A set of standard samples with a known protein concentration were established by diluting volumes of bovine serum albumin (BSA) (New England Biolabs) 1mg/ml with volumes of water in different ratios. These ranged from 0 to 500mg/ml. The test samples were diluted with H_20 in a ratio of 1:9. 10µl of each sample was added to a 96-well plate and all samples were analysed in triplicate. Bradford's reagent (Bio-Rad) was diluted with 1:4 with water and 200µl was added to each sample. The colour intensity was measured using a 595 filter with an automated reader. The mean absorbance value for each of the standard samples was plotted against the protein concentration using Cricket Graph software. The value of the equation $y=mx+c$ was calculated for the best-fit line.

The protein levels in the test samples were then determined using the equation:

(Protein Concentration (µg/µl) x 10)/1000 = (Mean Absorbance – *c*) / *m*

3.3.5 Western blotting

Proteins were separated using 6% or 8% Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels. Six percent gels were used in experiments that required the separation of large molecular weight proteins such as ATM, and 8% gels were used in experiments investigating the levels of small molecular weight proteins such as p21 and cleaved caspase 3. The gels were made using 27ml or 24.6 ml of high purity H_20 and 8ml or 10.7mls of polyacrylamide (Bio-Rad) (6% or 8% gels, respectively), plus 4ml 1M Tris-Biscine, 400µl 10%-SDS, 80µl N N N N -Tetra-methyl-ethylene-diamine (Temed) and 200µl 10% Ammonium persulphate (APS) (Bio-Rad). The wells of the gels were subsequently washed with running buffer (50ml of 1M Tris-Biscine, 5ml of 10% SDS and 450ml H_20)

The protein lysates were defrosted briefly at 37ºC and 30µg of each sample was aliquoted into microcentrifuge tubes. In the experiments that had involved the use of fixed numbers of cells per time point, the entire lysate (15µl) was used. Six microlitres of 4x sample buffer (0.25M Tris HCl ph 6.8, 8% SDS, 40% glycerol, 0.4M DTT and 0.04% Bromophenol blue) was added to each lysate and the proteins were denatured in a heat block at 100 ºC for 5 minutes and loaded into the wells of the gel using a Hamilton Syringe. A rainbow marker (Amersham) was run on each gel to indicate protein size.

Running buffer was added and electrophoresis was performed at 30mA for approximately 4 hours.

After electrophoresis, the gel was blotted onto a nitrocellulose filter (Gelman) at 200mA for 12 hours in an Hoeffer transblot electroblotter in blotting buffer (1400ml methanol (Riedel de Haen), 40.6 gm Tris, 203 gm Glycine made up to a volume of 7 litres with water.

After blotting the filter was stained with Ponceau stain (1% Ponceau Red in 3% (w/v) trichloroacetic acid) for 2 min on a rocker, and then de-stained briefly with water to visualise the blotted proteins. The filters were subsequently cut according to the size of the protein under investigation and were washed 3 times (ten minutes) with either Tris Buffered Saline Tween (TBST) (pH7.6), containing 10l of water, 24.2g Tris, 80g NaCl and 10ml (0.1%) of Tween 20, or Phospho Buffered Saline Tween (PBST), containing 10l of water, 1 pot of PBS tablets (Oxoid) and 10ml (0.1%) of Tween-20, in order to completely remove the stain. TBST was used for experiments that involved the assessment of phosphorylated proteins. The filters were then blocked in PBST or TBST with 5% milk at room temperature for 2 hours on a rocker and washed three times, prior to incubation at the appropriate time and temperature with the primary (1º) antibody diluted in milk block. After a further 3 washes filters were incubated with the appropriate horse-radish peroxidase-labelled anti-species antibody conjugate (secondary antibody) diluted in milk block for one hour. This was following by a further washing in PBST or TBST three times for 10 minutes. The blots were then developed using an enhanced chemiluminescence (ECL) detection system (Pharmacia) according to the manufacturer's instructions and exposed to X-omat film (Kodak).

The primary antibodies used for Western blotting in this project were as follows: rabbit antibody to serine 15 phosphorylated p53 (Sigma Aldrich), sheep antibody to p53 (donated by DP Lane, University of Dundee), rabbit antibody to serine 1981 phosphorylated ATM (Sigma Aldrich), mouse monoclonal ATM antibody (developed in our laboratory), mouse rabbit antibody to serine 966 phosphorylated structural maintenance of chromosome 1 (SMC1) (Bethyl Laboratories), rabbit antibody to SMC1 (Upstate), rabbit antibody to serine 343 phosphorylated Nbs1 (Santa Cruz) rabbit antibody to Nbs1 (Novus Biologicals), mouse monoclonal antibody to γH2AX, rabbit polyclonal antibody to p21 (Santa Cruz), mouse antibody to MDM2 (Donated by R Grand), mouse antibody to PUMA (Upstate), mouse antibodies to PARP1 (poly ADP ribose polymerase 1) (R&D systems), rabbit antibody to procaspase 7 and caspase 3 (New England, Biolabs), mouse monoclonal antibody to actin (Sigma Aldrich).

The secondary antibodies were goat polyclonal to mouse IgG (Sigma Aldrich), rabbit polyclonal to sheep IgG (DAKO) and porcine polyclonal to rabbit IgG (DAKO).

Optical density of protein bands was measured using Quantitiy One Imaging and Analysis Software (Bio-Rad) in order to quantify responses between tumours. Comparisons were made between samples on the same blot to control for any differences in antibody efficacy or exposure times. Changes in the level of a protein were expressed relative to any changes in either actin, or in the case of experiments studying phosphorylated proteins, relative to the level of the total un-phosphorylated protein.

3.4 Immunofluorescence

Immunofluorescence was used to assess the formation of γH2AX foci.

Buffers were prepared in advance. Pre-extraction buffer was made to a volume of 250mls (pH6.8) with Pipes (10mM), Sucrose (300mM), NaCl (20mM), $MgCl_2$ (3mM) and 0.5% Triton x-100. Fixation buffer consisted of 3.6% Paraformaldehyde in PBS. High purity water (300ml) was heated with 300µl NaCl and 10.8g paraformaldehyde and 3 PBS tablets (Oxoid) were added. After cooling, the pH of the buffer was adjusted to 7.2. This buffer should be stored at -20˚C.

Before usage, immunofluorescence slides were cleaned in 70% Ethanol / 1% Hydrochloric acid for 1 hour. They were subsequently washed x 4 for 20 minutes per wash in H_20 and dried thoroughly (37˚C). Poly-L-lysine was then placed on each well that was to be used and left for 20 minutes. Wells were washed with water and allowed to dry thoroughly (37˚C).

Approximately 1 x 10^5 cells were used per time point. Cells were transferred from culture plates into universal tubes and spun at 1000 rpm for 4 minutes. The majority of the supernatant was removed and the cell pellet was re-suspended in 30µl of residual supernatant. The cells were transferred to the wells on the slides and left to adhere for 20 minutes.

Slides were placed in extraction buffer for 5 minutes and subsequently fixed in ice-cold paraformaldehyde for 10 minutes. The slides were then washed x 3 (5minutes per wash) in Tris buffered saline (TBS) before being transferred into fetal calf serum (FCS) blocking solution (FCS diluted to 10% in TBS for 1 hour).

After washing three times for 5 minutes, excess TBS buffer was wiped from the wells and 10µl of the appropriate primary antibody (anti-phospho-Histone H2AX (Ser 139) (Upstate) diluted 1:500 in TBS) was added to each well. Slides were incubated for 1 hour in a dark moist box. After a further three washes slides, 30µl of secondary antibody, (Fluorescent conjugated Alexa 594 anti-mouse antibody diluted 1:1000 in TBS) was added to each well and slides were incubated for a further hour.

Slides were washed as previously and excess buffer removed from the slides. A DAPI/Vector shield mix (DAPI stain (1µg/ml in water) 1:4 Vectorshield) was made and 1 drop was placed over each well. Slides were covered with a coverslip, which was sealed with nail varnish, and stored in the dark at 4˚C. Slides were examined using a fluorescent microscope and images were stored using 'Open lab' software.

3.5 Fluorescent in situ hybridisation (FISH)

A cytospin was initially prepared for each sample. Up to 2x 10^7 thawed tumour cells were washed twice with PBS and subsequently re-suspended in between 5-7mls PBS. 2 drops of this cell suspension was then used to make a cytospin. Cells were subsequently fixed in a mixture of methanol and acetic acid (3:1) at 4ºC for one hour. The slides with the fixed cells were stored in the dark at -20ºC.

Slides were prepared for FISH by incubating them in 2xSSC buffer (Sodium chloride (0.3M), Tri -Sodium Citrate (Sigma) (0.03M)) for 30 minutes at 37ºC. They were subsequently washed in increasing concentrations of ethanol, 70%, 80% and 100% for 2 minutes each. After drying, the position of

the cells was marked on the slide and 10µl of hybridisation buffer (50% formamide plus 2XSSC) was placed over the cells using a cover slip, whose edges were sealed. Slides were heated for 5 minutes at 70ºC to allow denaturation. Commercial probes (Qbiogene) were denatured by heating to 96ºC for two minutes. This product contains an ATM probe that will bind to the *ATM* gene localised on the minimal region of deletion on chromosome 11q22-23, together with a chromosome 11 centromeric probe. The hybridisation buffer was removed from the slide and a new coverslip, on which with 5µl of the commercial probe mixture had been added, was placed over the cells. A seal was placed around the edge of the coverslip and the slides were heated to 70ºC for 5 minutes to allow co-denaturation. Slides were left in the dark overnight to allow probe hybridisation.

Following hybridisation, the slides were first placed in 2xSSC in order to remove the cover slips and then in a mixture of 0.4xSSC and NP40 at 65ºC for 5 minutes. After cooling in 2xSSC, Vectoshield (containing DAPI) was placed over the cells with a new coverslip.

The cells were visualised under a fluorescent microscope and the number of centromeric (green) probes and ATM (red) probes in each cell were counted. Examples of cells were photographed using ISIS software.

3.6 Clinical Data and Statistical Analysis

I collected clinical data including dates of birth, dates of diagnosis, gender, age at diagnosis, clinical stage at diagnosis, treatment history, and survival data from hospital case notes and recorded these in a CLL patient database.

The *IGVH* mutation status had been determined for a majority of CLL patients as part of independent studies. Tumours were classified as *IGVH* un-mutated if there was at least 98% concordance between the tumour DNA and the respective family sequence, and *IGVH* mutated if there was less than 98% concordance.

The Kaplan Meier method was used to compare the overall survival (OS) and treatment free survival (TFS) between subgroups of patients according to their genetic subgroup. The log rank test was used to compare the significance of any differences in survival between these subgroups. The Fisher's exact test was used to confirm any association between *ATM* mutation status and *IGVH* mutation status, the Chi-squared test to determine any associations between *ATM* mutations and clinical stage at diagnosis, and the Mann-Whitney U Test to determine any association between *ATM* mutations and age at diagnosis. The Kruskall-Wallis test was used to determine if there were significant differences between the median levels of PARP1 cleavage between the three genetic groups.

Multivariate analysis by Cox's proportional hazards regression was used to analyse the unselected cohort and assess the inter-dependence of various prognostic factors in order to determine whether *ATM* mutations could provide any independent information (The statistical analysis was done with the help of Dr Judith Powell, Department of Epidemiology and Public Health, University of Birmingham).

Results 1

The prevalence and nature of *ATM* mutations in CLL cohorts

4.1 Introduction

Although sporadic mutations in the *ATM* gene have been reported to occur in CLL tumours (Stankovic T *et al*, 1999, Schaffner C *et al*, 1999, Bullinger F *et al* 1999) their prevalence has not been established. Previous studies of *ATM* mutations in CLL have involved small cohorts of patients that have consisted primarily of patients with aggressive disease features, such as advanced stage disease (B or C) and un-mutated *IGVH* genes. In contrast, a number of large studies have investigated the prevalence of a deletion of chromosome 11q that leads to the loss of one copy of the *ATM* gene (Dohner H *et al*, 2000, Dewald GW *et al*. 2003). However, the precise relationship between mutations in the *ATM* gene and deletions of an *ATM* allele via loss of chromosome 11q in CLL tumours remains obscure. For example, there remains uncertainty regarding the proportion of tumours with an 11q deletion that also have an *ATM* mutation in the remaining allele.

The primary aims of the work described in this chapter, therefore, has been to establish the prevalence of *ATM* mutations within (1) a large unselected cohort of CLL tumours and (2) a selected cohort of CLL tumours, which all had a deletion of chromosome 11q. In addition, I have investigated the nature of the *ATM* sequence changes and their distribution across the *ATM* gene within the two CLL cohorts.

The majority of the *ATM* mutations that have been described in CLL tumours have been shown to be sporadic changes that were acquired within the tumour cell. One study has also reported the presence of germ-line *ATM* mutations in a small proportion of cases. Although numbers were small, these germ-line changes were present at a higher frequency in the CLL patients than had been previously reported to occur within the general population, suggesting the possibility of a causative role for *ATM* mutations in the development of CLL (Stankovic T *et al*, 2002). In addition, specific germ-line polymorphic alterations in the *ATM* gene sequence have been linked to the development of malignancies including CLL (Scott SP *et al*, 2002, Rudd MF *et al*, 2006). To date, however, there has been no direct comparison between the *ATM* sequence changes occurring in CLL patients with those occurring in a control population, which has used a screening technique designed to detect all changes present. Such an approach would allow the assessment of the potential of a causative role of *ATM* variants in CLL.

A secondary aim that is addressed in this chapter, therefore, has been to determine whether there are differences in either the nature or frequency of germ-line *ATM* sequence alterations between CLL patients and a control population.

4.2 Nature of the cohorts

4.2.1 Cohort of unselected CLL patients

To determine the prevalence of *ATM* mutations in unselected CLL patients, I established a cohort that was representative of the clinical CLL phenotype spectrum and was of sufficient size to provide adequate power to determine mutation prevalence. The cohort consisted of 155 patients with CLL that had attended a haematology outpatient clinic at either Selly Oak or Heartlands Hospitals between the dates of December 2001 and January 2003. As far as possible, all CLL patients attending one of these clinics during the study period were included and therefore the cohort included both newly diagnosed patients and also many patients that had originally been diagnosed with CLL at varying times prior to the study period.

4.2.2 Cohort of CLL patients with a deletion of chromosome 11q

To establish the prevalence of *ATM* mutations in the subgroup of CLL patients that all had loss of one copy of the *ATM* gene, I formed a second CLL cohort, selected by the presence of a deletion of chromosome 11q in their tumour cells. This cohort consisted of 53 CLL patients and the detection of the deletion of chromosome 11q by FISH in the tumour cells had been previously performed in diagnostic laboratories using an ATM probe. The patients originated from hospitals in the West Midlands, Hampshire and Leicestershire and included a mixture of recently diagnosed patients and those that been diagnosed some years previously.

Ten patients fulfilled the entry criteria for both cohorts and were, therefore, included in both patient groups. Thus, in total 198 CLL patients were analysed for *ATM* mutations in this study.

4.2.3 Cohort of control individuals

The control cohort consisted of 71 Caucasian healthy volunteers over 65 years in age and 90 anonymous individuals who were blood donors making a total of 161 controls.

4.3 Nature of *ATM* sequence changes in the CLL patients

Altogether 198 CLL tumours from the two cohorts were screened for *ATM* mutations by a combination of DHPLC and direct sequencing as described. At least one sequence change in the *ATM* gene was detected in 138 of these cases, and in 59 cases more than one change was present. There were sixty-nine different sequence alterations detected which included unique alterations present in a single individual and certain common changes, such as 5557G/A, occurring in 23% of the entire group. Fifty-six of the changes were present within the coding exonic sequence and thirteen within flanking intronic sequences. Sixty changes were nucleotide substitutions, eight were nucleotide deletions and one change was a nucleotide insertion.

4.3.1 Distinguishing mutations from polymorphisms

ATM is a highly polymorphic gene and distinguishing a mutation, which will lead to impaired function of the translated protein, from a polymorphism, with no functional consequences, is not always straightforward. Non-sense

changes result in the generation of a premature stop codon, either through an out of frame deletion or insertion or a nucleotide substitution that causes the direct formation of a stop codon. These changes are predicted to lead to a truncated protein and therefore are likely to represent a mutation. Mis-sense changes are due to nucleotide substitutions and are predicted to lead to an amino acid substitution in the translated protein. Depending on the site and nature of the change within the protein, such an amino acid change may or may not have consequences on the function of the translated protein. Alterations in the charge or polarity of an amino acid and / or the involvement of a residue that is conserved across species might be predicted to be more likely to have an effect on protein function. Other sequence changes can represent mutations by interfering with the process of exon splicing. Splicing is determined by the presence of consensus sequences that form donor and acceptor sites for RNA splicing and in certain cases also require additional splicing enhancer and silencer sequences. A sequence change at or near an intron / exon boundary can lead to disruption of the splicing process which can lead to skipping of a whole exon (Thortenson YR *et al*, 2003).

In this study, a sequence change was designated as a mutation if it:

a) Led to the generation of a premature stop codon (Non-sense change),
b) Caused an amino acid substitution (Mis-sense change) or in-frame deletion or insertion that had not been reported as polymorphism nor was present in 200 normal alleles, or
c) Had been previously reported to result in abnormal exon splicing

4.3.2 *ATM* mutations in the CLL patients

Using these criteria, 36 changes were categorised as mutations and 33 changes as polymorphisms in the CLL patients (Tables 4.1 and 4.2). Amongst the changes designated to be mutations there were 10 non-sense changes, 21 mis-sense changes in the coding sequence, one in-frame insertion, one in-frame deletion and 3 changes that were predicted to interfere with splicing.

4.3.2.1 Non-sense *ATM* mutations

There were six out of frame deletions in the coding sequence of the *ATM* gene (1058delGT, 3651delC, 5290delC, 6815delA, 7883del5, 8833del34), which would generate a downstream stop codon, and four nucleotide substitutions (1120C/T (Q374X), 2308G/T (E770X), 4591C/T (Q1531X) 8977C/T (R2993X)) leading to the direct generation of a stop codon. All these changes were unique in a single individual and, notably, the mutation 5290delC has been previously reported in AT patients.

4.3.2.2 Mis-sense *ATM* mutations

Twenty-one changes were classified as mis-sense mutations resulting from an nucleotide substitution which would be predicted to result in an amino acid substitution in the translated in protein (1009C/A, 2417T/G, 3383A/G, 3964C/A, 5041A/G, 5044G/T, 5224G/C, 5228C/T, 5882A/G, 5980A/G, 6067G/A, 6106T/A, 7047C/G, 7313C/A, 8056T/C, 8249T/C, 8600G/A, 8839A/T, 8861A/G, 9022C/T, 9023G/A). Each of the changes was present in a single tumour except for the 8249T/C and 5980A/G substitutions that were each detected in two CLL tumours. Two of the changes (5228C/T, 9022C/T)

Table 4.1 – Sequence changes designated as mutations in the *ATM* gene in the CLL patients

Nucleotide Change	Type of mutation	Protein Change	Number of Patients With Change	Conserved between Human and Mouse sequence	Present in Germ line	Previous reports of change
1009C/A	Mis-sense	R337S	1	Yes	No	Not reported
1058DelGT	Non-sense	Truncation	1	na	Yes	Reported (in this patient)
1120C/T	Non-sense	Q374X	1	na	No	Reported in AT patients
IVS16-10T/G	Splicing	Splice defect	1	na	nk	Reported in AT patients
2308G/T	Non-sense	E770X	1	na	No	Not reported
2417T/G	Mis-sense	L806W	1	Yes	No	Not reported
2929Ins9	Insertion	Insertion 3	1	na	nk	Not reported
3383A/G	Mis-sense	Q1128R	1	No	nk	Not reported
3651delC	Non-sense	Truncation	1	na	No	Not reported
3883Del3	Deletion	Deletion L	1	na	nk	Not reported
3964C/A	Mis-sense	L1322F	1	Yes	Yes	Not reported
4591C/T	Non-sense	Q1531X	1	na	nk	Not reported
5041A/G	Mis-sense	I1681V	1	No	No	Not reported
5044G/T	Mis-sense	D1682Y	1	Yes	No	Reported in T-PLL
5224G/C	Mis-sense	A1742P	1	na	No	Reported (in this patient)
5228C/T	Mis-sense	T1743I	1	Yes	No	Reported in AT patients
5290del C	Non-sense	Truncation	1	na	No	Reported in AT patients
5882A/G	Mis-sense	Y1961C	1	Yes	Yes	Not reported
5980A/G	Mis-sense	K1994E	2	Yes	No	Not reported
6067G/A	Mis-sense	G2023R	1	Yes	Yes	Breast Cancer, DLBCL
6106T/A	Mis-sense	Y2036N	1	Yes	No	Not reported
6815DelA	Non-sense	Truncation	1	na	No	Not reported
7047C/G	Mis-sense	C2349W	1	Yes	No	Not reported
7313C/A	Mis-sense	T2438K	1	Yes	nk	Not reported
7883del5	Non-sense	Truncation	1	na	No	Not reported
8056T/C	Mis-sense	F2686L	1	Yes	No	Not reported
8249T/C	Mis-sense	L2750S	2	Yes	Yes (1/2)	Not reported
8592C/T	Splicing	Splice defect	1	Na	Nk	Reported, splicing variant
8600G/A	Mis-sense	G2867E	1	Yes	No	Reported (in this patient)
IVS62-1G/T	Splicing	Splice defect	1	na	nk	Reported in AT patient
8839A/T	Mis-sense	T2946S	1	Yes	No	Reported (in this patient)
8833Del34	Non-sense	Truncation	1	na	No	Not reported
8861A/G	Mis-sense	Y2954C	1	Yes	No	Not reported
8977C/T	Non-sense	R2993X	1	na	nk	Not reported
9022C/T	Mis-sense	R3008C	1	Yes	No	Reported in AT patients
9023G/A	Mis-sense	R3008H	1	Yes	No	AT patients, MCL, CLL

Table 4.2 - Sequence changes designated as polymorphisms in the *ATM* gene in the CLL patients

Nucleotide Change	Protein Change	Number of Patients With the Change	Previous reports of change
146C/G	S49C	1	Known polymorphism
378T/A	D126E	3	Known polymorphism
IVS8+38T/C	Intron	2	Known polymorphism
795T/C	I265I	2	Known polymorphism
998C/T	S332F	1	Known polymorphism
1176C/G	G392G	1	Known polymorphism
1254A/G	E418E	1	Known polymorphism
1986T/C	F662F	1	Known polymorphism
2119T/C	S707P	6	Known polymorphism
IVS19-17G/T	Intron	1	Known polymorphism
2572T/C	F858L	9	Known polymorphism
2805G/C	T935T	1	Known polymorphism
3161C/G	P1054R	7	Known polymorphism
IVS25-15delA	Intron	26	Known polymorphism
IVS29-33C/T	Intron	1	Intronic location (Not reported)
4138C/T	H1380Y	2	Known polymorphism
4258C/T	L1420F	11	Known polymorphism
4578C/T	P1526P	15	Known polymorphism
IVS33+25A/G	Intron	1	Intronic location (Not reported)
4802G/A	S1601N	1	Known polymorphism
5071A/C	S1691R	1	Known polymorphism
IVS38-8T/C	Intron	4	Known polymorphism
5352C/T	N1784N	1	No amino acid change (Not reported)
5557G/A	D1835N	46	Known polymorphism
5558A/T	D1853V	1	Known polymorphism
5793T/C	A1931A	5	Known polymorphism
5821G/C	V1941L	2	Present in controls (Not reported)
6820G/A	A2274T	1	Known polymorphism
IVS52+4A/G	Intron	1	Intronic location (Not reported)
IVS54+8G/T	Intron	1	Present in controls (Not reported)
IVS61+9T/G	Intron	1	Intronic location (Not reported)
IVS62+8A/C	Intron	19	Known polymorphism
9200C/G	3'UTR	4	Known polymorphism

have been previously found in AT patients and four of the changes have been reported in association with haematological tumours, (5044G/C (T-PLL), 6067G/A (DLBCL), 9022C/T (T-PLL and MCL) and 9023G/A (MCL and CLL)).

In 19 of the 21 mis-sense changes that were predicted to lead to amino acid substitutions the amino acid residue involved was one that was conserved between the human and mouse *ATM* gene sequence (1009C/A (R337S), 2417T/G (L806W), 3964C/A (L1322F), 5044G/T (D1682Y), 5224G/C (A1742P), 5228C/T (T1743I), 5882A/G (Y1961C), 5980A/G (K1994E), 6067G/A (G2023R), 6106T/A (Y2036N), 7047C/G (C2349W), 7313C/A (T2438K), 8056T/C (F2686L), 8249T/C (L2750S), 8600G/A (G2867E), 8839A/T (T2946S), 8861A/G (Y2954C), 9022C/T (R3008C), 9023G/A (R3008H)). This provides further support that these sequence changes represent mutations and that an alteration in one of these conserved residues will affect the function or stability of the ATM protein in the cell.

Two of the sequence changes (3383A/G (Q1128R) and 5041A/G (I1681V)) involved the substitution of an amino acid that was not conserved between the mouse and human *ATM* sequence. Nevertheless, these changes were still considered to be mutations as they fulfilled the 'mutation criteria' that had been defined for this study. For the change 3383A/G (Q1128R), the mutation would result in the substitution of one charged hydrophilic residue for another, whereas the change 5041A/G (I1681V) would result in the exchange of uncharged hydrophobic residues. Interestingly, this second sequence change occurred in combination with the 5044G/T (D1682Y) mutation, involving the conserved adjacent amino acid residue in the same individual. As such it is possible that it is the combination of the two amino acid substitutions which

impacts on ATM function. The 5041A/G sequence change was also shown to be an acquired alteration that was absent from the patient's germ-line DNA, thus providing further evidence that this change was indeed a mutation rather than a polymorphism. Unfortunately, there was no germ-line material available on the patients with the 3383A/G sequence alteration so it was not possible to determine if this represented an acquired or germ-line change.

4.3.2.3 Other *ATM* mutations

In addition to the nucleotide substitutions there was one mutation that resulted in an in-frame change in the coding sequence due to an insertion of nine nucleotides (2929Ins9), which would be predicted to result in an insertion of three amino acids, and one mutation that resulted in an in-frame change due to a deletion of three nucleotides (3883Del3), which would be predicted to result in a deletion of an amino acid in the translated protein. Neither of these changes had been previously reported.

Three of the sequence changes were classified as mutations because they have been previously reported to cause abnormal exon splicing (IVS16-10T/G, IVS62-1G/T, 8592C/T). The changes IVS16-10T/G and IVS62-1G/T have been reported in AT patients. The IVS16-10T/G change, leads to an insertion of three amino acids and results in the generation of a new splice acceptor site (Telatar M *et al*, 1998) and the IVS62-1G/T change is known to cause aberrant exon splicing (Sandoval N *et al*, 1999). The 8592C/T sequence change has not been reported in AT patients but has been previously detected in association with cancer and is predicted to result in the

disruption of an exon splicing enhancer sequence (Thorstenson Y *et al*, 2003).

4.3.3 *ATM* polymorphic changes in the CLL patients

Thirty-three changes were classified as polymorphisms including 23 within the coding sequence (146C/G, 378T/A, 795T/C, 998C/T, 1176C/G, 1254A/G, 1986T/C, 2119T/C, 2572T/C, 2805G/C, 3161C/G, 4138C/T, 4258C/T, 4578C/T, 4802G/A, 5071A/C, 5352C/T, 5557G/A, 5558A/T, 5793T/C, 5821G/C, 6820G/A, 9200C/G) and ten within flanking intronic sequences (IVS8+38T/C, IVS19-17G/T, IVS25-15delA, IVS29-33C/T, IVS33+25A/G, IVS38-8T/C, IVS52+4A/G, IVS54+8G/T, IVS61+9T/G, IVS62+8A/C). Sixteen of these changes were detected in multiple CLL patients and twenty-six of the changes have been reported to be polymorphisms in the literature (Table 4.2). Two changes in the coding sequence (5352C/T, 5821G/C) and five within introns (IVS29-33C/T, IVS33+25A/G, IVS52+4A/G, IVS54+8G/T, IVS61+9T/G) were also classified as polymorphisms in this study, although they had never been previously reported as such. The 5352C/T is a silent change that would not result in a change of amino acid in the translated protein. The 5821G/C was initially considered to be a mutation but was subsequently re-classified as a polymorphism because it was not only detected in two CLL patients but also within an individual from the control population. Furthermore, this change was present in the germ-line as well as within the tumour material in both CLL patients. The change IVS54+8G/T has been reported in the germ-line of patients with breast cancer but since it was also detected in two individuals from the control population in this study it was

classified as a polymorphic change (Teraoka S *et al*, 2001). The remaining intronic changes were also absent from the control population. However, although they had not been previously reported, since they were located within the intronic sequence and unlikely to impact on the protein they were designated as polymorphisms rather than mutations.

4.4 Prevalence and distribution of *ATM* mutations in the cohorts

Having screened the tumour samples for sequence changes in the *ATM* gene and categorised the changes as mutations and polymorphisms, I was then able to determine the prevalence of the *ATM* mutations in each of the two CLL patient cohorts.

4.4.1 The unselected cohort of 155 CLL patients

There were twenty-two *ATM* mutations in eighteen of the CLL tumours from the unselected cohort of 155 patients and this represented a prevalence of CLL tumours carrying *ATM* mutations of 12%. Five mutations were non-sense changes, fourteen mutations were mis-sense changes, one mutation was an in-frame insertion, and two mutations were predicted to interfere with exon splicing.

In seven tumours there was a predicted loss of both functional *ATM* alleles; in three cases (CLLs 96, 11 and 77) due to more than one *ATM* mutation detected and in four cases (CLLs 07, 15, 57 and 152) due to an *ATM* mutation in combination with a deletion of the remaining *ATM* allele on chromosome 11q. In the remaining ten tumours a single *ATM* mutation was detected but

there was no loss of the remaining *ATM* allele through a chromosome 11q deletion (Table 4.3).

In this cohort the mutations were distributed across the coding sequence of the gene and even when considering only the mis-sense changes there was an absence of clear clustering within the conserved C terminal region of the gene including the kinase and FAT domains. There were 3 mutations (9022C/T (R3008C), 8839A/T (T2946S) and 8600G/A (G2867E)) in the kinase domain and 3 mutations (5980A/G (K1994E), 7047C/G (C2349W) and 7313C/A (T2438K)) in the FAT domain. The distribution of all the 14 mis-sense changes plus the in-frame insertion from this cohort is shown in Figure 4.1. Interestingly, three of the changes within these two functional domains were in tumours that also had a deletion of chromosome 11q and were therefore common to both cohorts.

4.4.2 The selected CLL cohort of 53 tumours

I detected mutations in the *ATM* gene in 20 out of 53 tumours in the cohort of tumours that all had loss of one *ATM* allele, indicating a prevalence of 38% (Table 4.4). Six of the mutations were predicted to lead to a truncated protein (CLLs 166, 183, 178, 198, 57, 170), one mutation was localised at an exon-intron boundary and had previously been reported to result in abnormal exon splicing (CLL 192), one mutation was an in-frame deletion (CLL180) and the remaining twelve mutations were mis-sense changes. All the mis-sense changes detected in this cohort involved residues that were conserved across species.

Table 4.3 - *ATM* mutations in unselected cohort of 155 CLL tumours

Tumour	Nucleotide change	Protein change	Mutation in germ-line	Conserved between human and mouse sequence	Loss of heterozygosity through a deletion of chromosome 11q
CLL77*	1058delGT 5224G/C	Truncated A1742P	Yes No	NA Conserved	No
CLL96*	2929ins9 5041A/G 5044G/T	Inframe insertion I1681V D1682Y	No No No	NA Not conserved Conserved	No
CLL11*	7313C/A 8833del34	T2438K Truncated	No No	Conserved NA	No
CLL57*[1]	2308G/T	E770X	No	NA	Yes
CLL15*[1]	7047C/G	C2349W	No	Conserved	Yes
CLL07*[1]	8600G/A	G2867E	No	Conserved	Yes
CLL152*[1]	8839A/T	T2946S	No	Conserved	Yes
CLL27	3383A/G	Q1128R	NK	Not conserved	No
CLL75	1009C/A	R337S	No	Conserved	No
CLL69	6815delA	Truncated	No	NA	No
CLL119	9022C/T	R3008C	No	Conserved	No
CLL93	5290del C	Truncated	No	NA	No
CLL92	5980A/G	K1994E	No	Conserved	No
CLL124	5228C/T	T1743I	No	Conserved	No
CLL84	8592C/T	Splicing defect	NK	NA	No
CLL113	3964C/A	L1322F	Yes	Conserved	No
CLL147	IVS16-10T/G	Splicing defect	NK	NA	NK
CLL81	5882A/G	Y1961C	Yes	Conserved	No

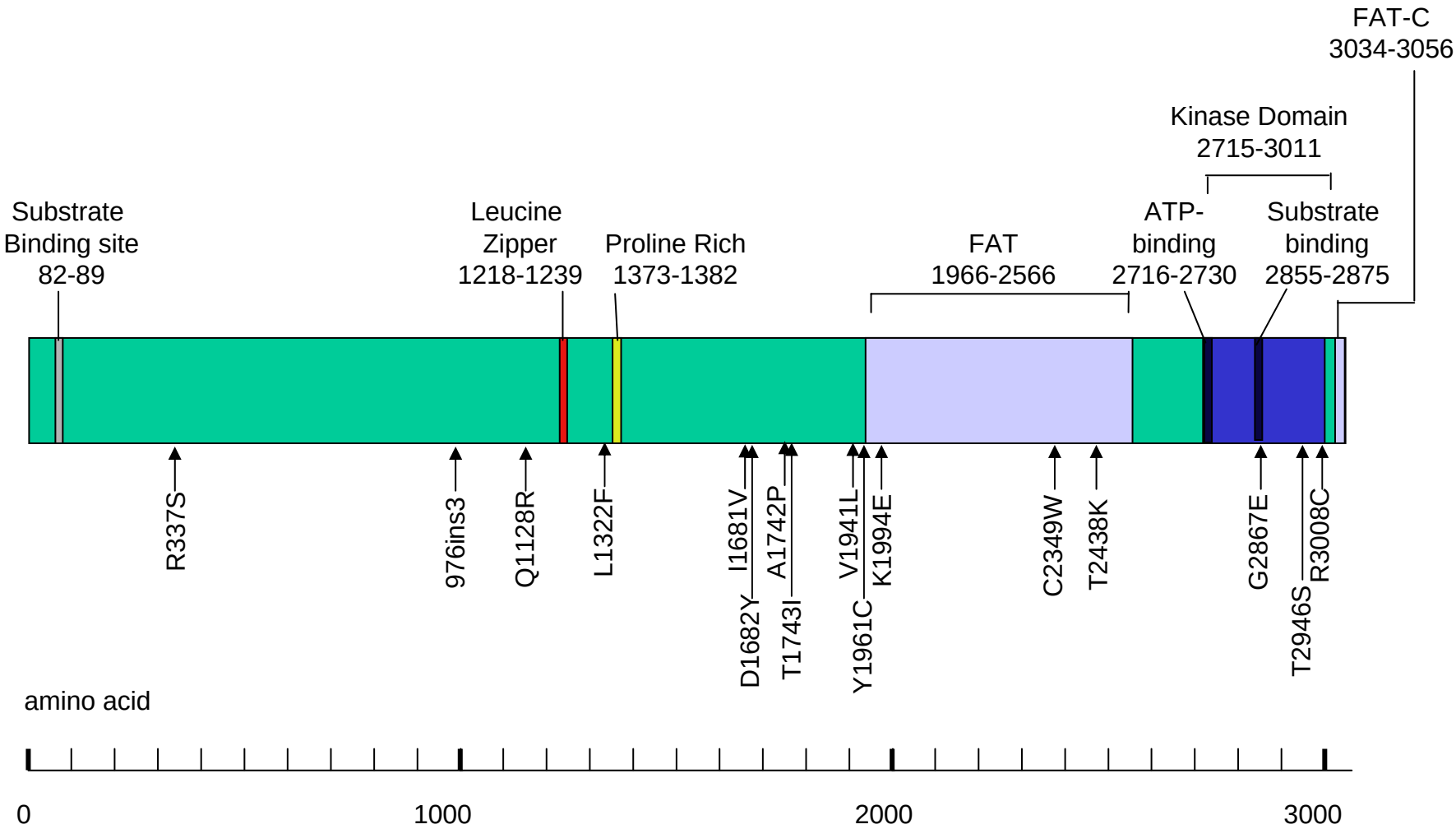
[1] Denotes tumours common to both cohorts

***** Denotes tumours with loss of both functional *ATM* alleles

Figure 4.1

The mis-sense changes detected in the unselected cohort of 155 patients were distributed across the whole protein and were not clustered within the functional domains.

Figure 4.1


FAT-C
3034-3056
Kinase Domain
2715-3011
Substrate
Binding site
82-89
Leucine
Zipper
1218-1239
Proline Rich
1373-1382
FAT
1966-2566
ATP-
binding
2716-2730
Substrate
binding
2855-2875
R337S
976ins3
Q1128R
L1322F
I1681V
D1682Y
A1742P
T1743I
V1941L
Y1961C
K1994E
C2349W
T2438K
G2867E
T2946S
R3008C
amino acid
0
1000
2000
3000

In each tumour there was only one *ATM* mutation detected but since all the tumours had loss of heterozygosity at the *ATM* gene locus, all 20 tumours with an *ATM* mutation would be predicted to have lost both functional alleles. In the remaining 33 tumours, no mutation was detected in the second *ATM* allele and therefore these tumours were heterozygous at the *ATM* gene as a result of a single allele deletion.

In contrast to the unselected cohort, within this cohort of tumours that all had a monoallelic loss of an *ATM* allele through a deletion of chromosome 11q, there was a tendency for the sequence changes to be clustered towards the C terminal region of the gene most particularly when only the missense changes were considered. Six mis-sense changes were located in the kinase domain (9023G/A (R3008H), 8861A/G (Y2954C), 8839A/T (T2947S), 8600G/A (G2867E) and 8249T/C (L2750S) (x2)), and four within the FAT domain (7047C/G (C2349W), 6106T/A (Y2036N), 6067G/A (G2023R) and 5980A/G (K1994E)). The distribution of the 12 mis-sense changes and the in-frame deletion is shown in figure 4.2.

4.4.3 Comparison between the two CLL cohorts

In this study, *ATM* mutations were greater than three fold more common in the selected cohort of tumours, which all had an 11q deletion, compared to the unselected CLL tumours. This suggests that either loss of one allele at the *ATM* locus predisposes to the development of *ATM* mutations, or that those tumours with *ATM* mutations are at risk of developing a chromosome 11q deletion. The nature of the mutations was similar in both cohorts, with mis-sense changes being the most common genetic alteration. However, the

Table 4.4 - *ATM* mutations in selected cohort of 53 CLL patients with a deletion of chromosome 11q

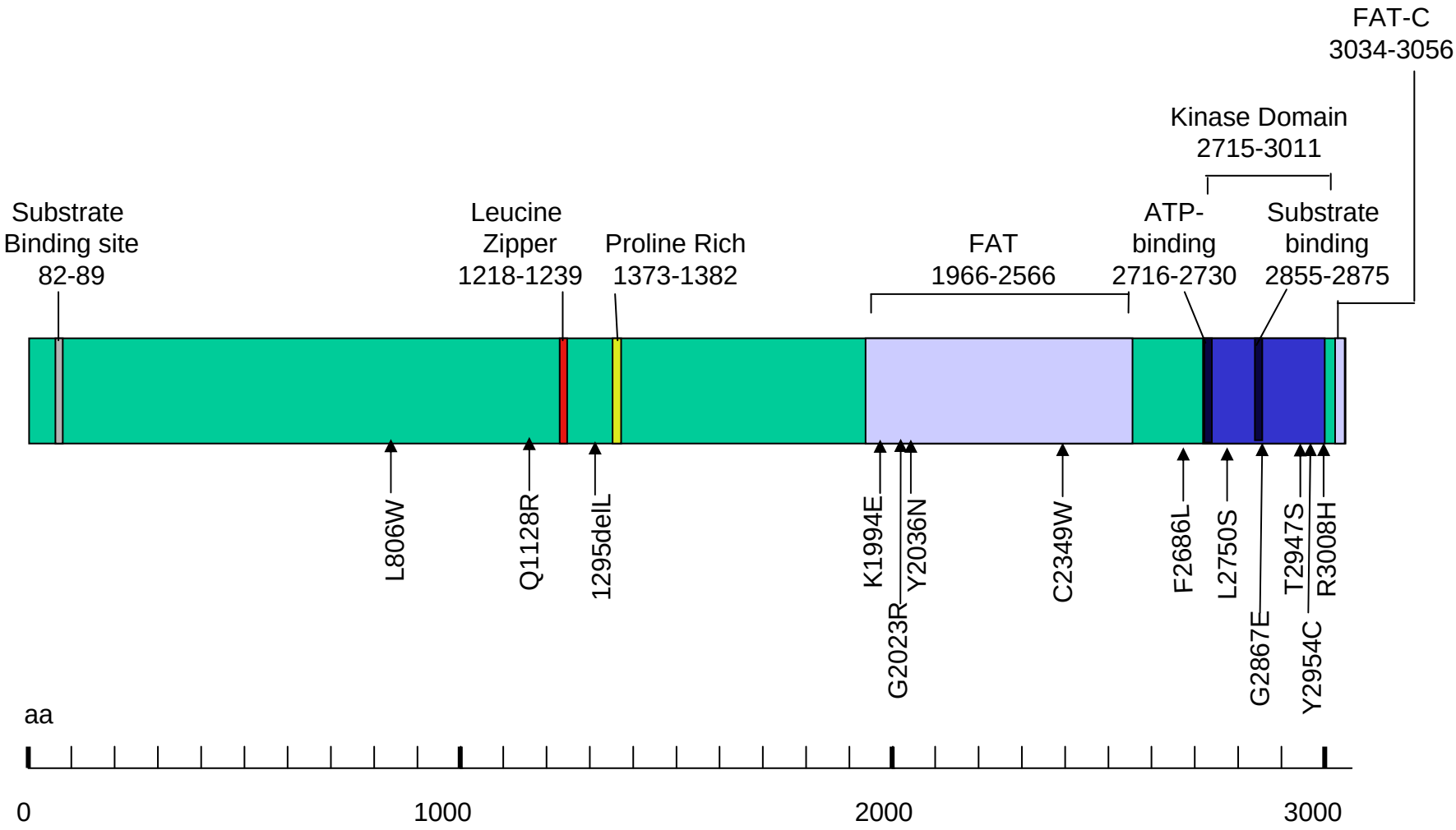
Tumour	Nucleotide Change	Protein Change	Mutation in germline	Conservation between human and mouse
CLL189	9023G/A	R3008H	No	Conserved
CLL166	8977C/T	R2993X	ND	NA
CLL174	8861A/G	Y2954C	No	Conserved
CLL152[1]	8839A/T	T2947S	No	Conserved
CLL192	IVS62-1G/T	Splice defect	No	NA
CLL07[1]	8600G/A	G2867E	No	Conserved
CLL200	8249T/C	L2750S	Yes	Conserved
CLL182	8249T/C	L2750S	ND	Conserved
CLL193	8056T/C	F2686L	No	Conserved
CLL183	7883del5	Truncated	ND	NA
CLL15[1]	7047C/G	C2349W	No	Conserved
CLL162	6106T/A	Y2036N	No	Conserved
CLL203	6067G/A	G2023R	Yes	Conserved
CLL169	5980A/G	K1994E	No	Conserved
CLL178	4591C/T	Q1531X	ND	NA
CLL180	3883del3	Deletion L	ND	NA
CLL198	3649delC	Truncated	No	NA
CLL173	2417T/G	L806W	No	Conserved
CLL57[1]	2308G/T	E770X	No	NA
CLL170	1120C/T	Q374X	No	NA

[1] Denotes tumours common to both cohorts

Figure 4.2

There was clustering of the predicted mis-sense changes across the ATM protein in the cohort of tumours that all had a deletion of chromosome 11q. There were six mis-sense mutations within the kinase domain and four mutations detected within the FAT domain of the protein.

Figure 4.2


FAT-C
3034-3056
Kinase Domain
2715-3011
Substrate
Binding site
82-89
Leucine
Zipper
1218-1239
Proline Rich
1373-1382
FAT
1966-2566
ATP-
binding
2716-2730
Substrate
binding
2855-2875
L806W
Q1128R
1295delL
K1994E
G2023R
Y2036N
C2349W
F2686L
L2750S
G2867E
T2947S
Y2954C
R3008H
aa
0
1000
2000
3000

distribution of the mutations varied between the two cohorts. The mis-sense changes in the unselected cohort were distributed across the coding sequence whereas the mis-sense changes in the 11q deleted cohort were clustered at the C- terminal part of the gene, in a pattern reminiscent to that reported in T-PLL, a tumour that is also characterised by loss of heterozygosity at the *ATM* locus.

There is marked homology of the ATM kinase domain, FAT and FATc domains between PIKK family members. The conservation of the gene sequence in these domains suggests that an alteration of even a single amino acid may be poorly tolerated and have an impact on the function of the protein.

My results show that sporadic mutations in the conserved kinase and FAT domains are invariably accompanied by loss of heterozygosity in CLL tumours. In the absence of such loss of heterozygosity, *ATM* mutations may fail to provide the tumour cell with any 'proliferative' or 'survival' advantage because the wild type allele is sufficient for normal ATM activity. By comparison, mutations located outside these domains might have a different mechanism of action. The function of the majority of the N-terminal two thirds of the gene appears to be related to the tertiary structure of the protein and, therefore, mutations in this region might affect the stability of the protein in the cell and it is possible that a pathogenic effect might occur in the absence of loss of the remaining *ATM* allele.

4.5 Frequency of sequence changes in CLL patients and controls

A secondary aim described in this chapter was to study the relationship between the frequency of germ-line *ATM* sequence changes between CLL patients and controls. The presence of a higher frequency of germ-line *ATM* mutations in CLL patients compared to controls could indicate that heterozygous mutations in *ATM* act as a predisposing factor for the development of CLL. Furthermore, it is also possible that *ATM* polymorphisms could contribute to the development of CLL and an increased association of a polymorphism with CLL patients compared to controls would support this concept. (The DHPLC mutation screening of the *ATM* gene in the control population was performed by technicians in our laboratory.)

4.5.1 Germ-line *ATM* mutations in CLL patients

I obtained germ-line material from 26 CLL patients with identified *ATM* mutations and in these cases it was therefore possible to determine whether a mutation was sporadic and acquired in tumour cells or whether the patient was a heterozygous carrier of a germ-line mutation. Twenty-three of the *ATM* mutations detected in patients' tumour cells from 21 patients, were shown to be sporadic and had thus been acquired during some stage of tumorigenesis (1009C/A, 1120C/T 2308G/T 2417T/G, 3651delG, 5041A/G, 5044G/T, 5224G/C, 5228C/T 5290delC, 5980A/G, 6106T/A, 6815DelA, 7047C/G, 7313C/A, 8056T/C, 8600G/A, 8833Del34, 8861A/G, 8839A/T, 9022C/T, 9023G/A, IVS62-1G/T). The confirmation of the acquired nature of these sequence changes provides further evidence that they are indeed likely to represent mutations with a pathologic effect.

Five of the *ATM* sequence changes classified as mutations in this study in the CLL tumour cohorts were also present in the same patient's germ line namely, 1058DelGT, 3964C/A (L1322F), 5882A/G (Y1961C), 6067G/A (G2023R) and 8249T/C (L2750S).

4.5.2 Germ-line *ATM* mutations in the control population

There were 8 sequence changes (162T/C (Y54Y), 609C/T (D203D), 1727T/C (I576T), 3419A/G (N1140S), 4167A/G (T1389T), 4980C/T (N1660N), 7390T/C (C2464R), IVS49+13insT) that were detected in the control population that were absent from the CLL population and had not been previously reported as a polymorphism. Four of the changes (162T/C (Y54Y), 609C/T (D203D), 4167A/G (T1389T), 4980C/T (N1660N)) would not result in an amino acid substitution in the translated protein and have not been reported to affect splicing and therefore these changes almost certainly represent rare polymorphisms. The sequence alterations IVS49+13insT has not been previously reported but due to its intronic location is likely to be a polymorphism. The sequence change 3419A/G (N1140S) was present in four of the controls and therefore, although this alteration had not been previously reported, due to this high frequency I considered that it was more likely to be a polymorphism rather than a mutation. The remaining two mis-sense changes (1727T/C (I576T), 7390T/C (C2464R)) were present in a single individual and were designated as germ-line *ATM* mutations. The 7390T/C is located within the FAT domain in the *ATM* gene and has been previously reported in patients with breast cancer, thus increasing the likelihood that this is a true mutation (Teroka S *et al*, 2001). By comparison, the 1727T/C

sequence change is not located within any known functional ATM domain and has never been previously reported, however this change does involve a residue that is conserved between human and mouse species.

Altogether, therefore, I found that 2 out of 161 (1.2%) controls and 5 out of 198 (2.5%) CLL patients had a germ-line *ATM* mutation (Table 4.5). These differences were not statistically significant (Chi-squared test). The small number of patients with germ-line *ATM* mutations detected in this study would indicate that a much larger study would be required to assess whether there was indeed a true difference between the two populations. The frequency of heterozygous *ATM* mutation carriers in the UK population has previously been estimated to be in the order of 0.5% and the frequency in the CLL patients in the previous study of 50 patients was 6% (3/50) (Stankovic *et al*, 1998, Stankovic *et al*, 2002). These estimates had indicated that there would be the possibility of detecting a significant difference between the two populations by a case-control comparison, where the size of those two populations was in the order that were studied in this project. However, this did not prove possible due to the lower frequency of germ-line *ATM* mutations detected in the CLL patients in my study.

There were several CLL patients where mutations were detected in the tumour cells but where there was no germ-line material available in this study. These mutations could have potentially represented germ-line changes and could therefore have resulted in an underestimation of germ-line mutations in the patient population. Alternatively, it is possible that some of the changes categorised as germ-line mutations in both patients and controls could have actually represented rare polymorphisms and this particularly applies to the

Table 4.5 – Germ-line *ATM* mutations in CLL patients and controls

Sequence Change	Protein Change	CLL patient or Control	Domain	Conserved between human and mouse
1058DelGT	Truncation	CLL patient	n/a	n/a
1727T/C	I576T	Control	None	Yes
3964C/A	L1322F	CLL patient	None	Yes
5882A/G	Y1961C	CLL patient	None	Yes
6067G/A	G2023R	CLL patient	FAT	Yes
7390T/C	C2464R	Control	FAT	Yes
8249T/C	L2750S	CLL patient	Kinase	Yes

mis-sense changes located outside of any functional *ATM* gene domains. It can be difficult to classify germ-line sequence alterations and, ideally, one would like to test the consequences of any potential germ-line mutations using an in vitro modelling system, as discussed later.

4.5.3 *ATM* polymorphisms in the CLL patients and controls

I also compared the allelic frequency of polymorphic changes between the unselected CLL patient cohort and the control population (Table 4.6). I was aware that in order to detect a potentially significant association of any individual polymorphism with the CLL patients it is likely to require larger populations than studied here, unless there is a very marked difference. However, given the size of the *ATM* gene, a large case-control study to address the total *ATM* allelic differences between a control and CLL population is a longer-term project. Therefore, the purpose of this part of the study was to provide a preliminary observation that could hopefully aid large population based comparisons of the frequency of specific *ATM* variants.

The polymorphisms in the CLL patients were detected in the tumour cells rather than in germ-line material and I made the assumption that these polymorphisms would also be present in the patients' germ-line. Since all the tumours with an 11q deletion had loss of one *ATM* allele, the 53 11q-deleted tumours were not included in this comparison due to their potential to negatively bias the frequency of a polymorphism in the CLL patients. Therefore, a comparison was made between 145 CLL patients and 161 controls. By using the DHPLC technique, there was also the theoretical possibility of underestimating any polymorphism that was a homozygous

Table 4.6 – Comparison of the frequency of polymorphisms in CLL patients and control individuals

Sequence Change	Amino Acid change	Frequency in 145 CLL patients	CLL Allelic Frequency (n/290)	Frequency in 161 controls	Control Allelic Frequency (n/322)	Ratio CLL / Control
146C/G	S49C	1/145	0.0034	2/161	0.0062	0.55
162T/C	Y54Y	0/145	0	1/161	0.0031	0
609C/T	D203D	0/145	0	1/161	0.0031	0
378T/A	D126E	2/145	0.0068	0/161	0	na
998C/T	S332F	1/145	0.0034	0/161	0	na
1176C/G	G393G	1/145	0.0034	0/161	0	na
1254A/G	E418E	1/145	0.0034	0/161	0	na
2119T/C	S707P	4/145	0.014	7/161	0.022	0.64
2572T/C	F858L	6/145	0.021	5/161	0.016	1.3
2805G/C	T935T	1/145	0.0034	0/161	0	na
3161C/G	P1054R	7/145	0.024	12/161	0.037	0.64
3419A/G	N1140S	0/145	0	4/161	0.012	0
4138C/T	H1380Y	1/145	0.0034	0/161	0	na
4167A/G	T1389T	0/145	0	1/161	0.0031	0
4258C/T	L1420F	8/145	0.028	9/161	0.028	1
4578C/T	P1526P	11/145	0.038	14/161	0.043	0.88
4980C/T	N1660N	0/145	0	1/161	0.0031	0
5071A/C	S1691R	0/145	0	2/161	0.0062	0
5352C/T	N1734N	1/145	0.0034	0/161	0	na
5557G/C	D1853N	33/145	0.11	66/161	0.20	0.55
5558A/T	D1853V	1/145	0.0034	0/161	0	na
5793T/C	A1931A	4/145	0.014	1/161	0.0031	4.5
5821G/C	V1941L	2/145	0.0068	1/161	0.0031	2.2
6820G/A	A2274T	1/145	0.0034	2/161	0.0062	0.55
IVS8+38T/C		1/145	0.0034	0/161	0	na
IVS19-17G/T		1/145	0.0034	0/161	0	na
IVS21-22 del T		0/145	0	4/161	0.012	0
IVS25-15delA		26/145	0.089	18/161	0.056	1.59
IVS29-33C/T		1/145	0.0034	0/161	0	na
IVS38-15G/C		0/145	0	1/161	0.0031	0
IVS38-8T/C		3/145	0.010	11/161	0.034	0.29
IVS49+13insT		0/145	0	1/161	0.0031	0
IVS52+4A/G		1/145	0.0034	0/161	0	na
IVS54+8G/T		1/145	0.0034	2/161	0.0062	0.55
IVS61+9T/G		1/145	0.0034	0/161	0	na
IVS62+8A/C		15/145	0.052	6/161	0.019	2.74
3'UTR 9200C/G		3/145	0.010	2/161	0.0062	1.61

variant in an individual. This would mainly apply to the most common sequence changes such as 5557G/A, and any effect would be expected to impact equally on patient and control groups.

For the majority of polymorphisms the frequency was comparable between the CLL patients and the controls (Table 4.6). Thirteen of the changes were absent from the control population and only present in the CLL population but in all cases they were only present in 1 or 2 CLL patients. Four of these polymorphisms (378T/A, 4138C/T, IVS8+38T/C and IVS19-17G/T) were associated with ethnic origin and the polymorphisms were found in CLL patients of Afro-Caribbean ethnicity, whereas no individuals were known to be Afro-Caribbean amongst the control population. By contrast, nine polymorphisms were present in the controls that were absent from the patient population. Once again the majority of these changes were only present in 1 or 2 controls suggesting that this is likely to be a chance effect. Two changes (3419A/G and IVS21-22delT) were present in 4 controls and no CLL patients but it is unlikely that the absence of a polymorphism could have any pathological effect and therefore this is also likely to be a chance association. Fifteen changes were present in both patient and control populations and for these cases the CLL to Control frequency ratio ranged from 0.29 to 4.5 (Table 4.6). Six changes were more common in CLLs than controls, namely 2572T/C (F585L) (ratio 1.3), 5793T/C (A1931A) (ratio 4.5), 5821G/C (ratio 2.2), IVS25-15delA (ratio 1.59), IVS62+8A/C (ratio 2.74) and 9200C/G (ratio 1.61). The 5793T/C, 5821G/C and 9200C/G sequence changes were each only present in up to 5 individuals and therefore any potential significance is hard to interpret. Of the remaining three polymorphisms only the IVS62+8A/C change

was present at a statistically significantly higher level in the CLL patients compared to controls (Chi-squared text, X^2 = 4.24 [0.01>p< 0.05]). This intronic change has not been previously found to be associated with malignancy but due to its location there is a possibility that this change could interfere with exon splicing.

Interestingly the 2572T/C (F585L), which occurred in 6 patients and 5 controls in this study, has been previously found to be significantly associated with CLLs compared to controls in a much larger case-control study using SNP arrays (Rudd MF *et al*, 2006). This same study also found an association between the 3161C/G (P1045R) change and CLL, but in my project this alteration was actually more common in the control population.

4.6 Conclusion

By analysing the two large CLL cohorts I have been able to show that *ATM* mutations are present in 12% of unselected CLL tumours and in 38% of tumours that have all lost one *ATM* allele through a chromosomal 11q deletion. Thus, the presence of loss of heterozygosity at the *ATM* locus is associated with a greater than three-fold increased frequency of *ATM* mutations in CLL tumours. The majority of these mutations were mis-sense changes and, interestingly, whilst there was no clustering pattern of mis-sense mutations observed within the unselected CLL tumours, within the tumours with loss of heterozygosity at the *ATM* locus, mis-sense *ATM* mutations showed a tendency to cluster within functional domains of the *ATM* gene at the C-terminus in this study. This latter pattern is reminiscent of the pattern

seen in T-PLL and MCL, whereas the former pattern resembles that which has been previously described in CLL series.

Most of the *ATM* mutations described here were acquired sporadic changes but there were a small number that represented germ-line changes. There was a small increase in the frequency of germ-line *ATM* mutations in the CLL patients by comparison to a control population but this was not statistically significant. A comparison of allelic variants, in this study, showed that the IVS62+8A/C change was more common in the CLL patients than in the control population.

Results 2

The clinical consequences and time of acquisition of *ATM* mutations in CLL patients

5.1 Introduction

A key aspect of my project has been to assess the consequences of *ATM* mutations on clinical outcome in CLL patients. Previously, *ATM* mutations have been found to be associated with certain poor prognostic features in CLL patients such as advanced stage and unmutated *IGVH* genes (Stankovic T *et al*, 2002, Pettitt AR *et al*, 2001) but their direct clinical impact has never been determined. By comparison, the detrimental effect on clinical outcome in CLL patients as a result of an 11q deletion, which results in the mono-allelic loss of a number of genes including *ATM*, is well established (Dohner H *et al*, 1999, Stilgenbauer S *et al*, 1996). Interestingly, it also remains unknown whether, within the subgroup of CLL patients that have an 11q deletion, there is a further impact on clinical outcome in those patients, which in addition to loss of one *ATM* allele, also have a mutation in the second *ATM* allele.

In order to compare the potential effect of a biological marker in a heterogeneous group of patients one can measure outcome parameters from diagnosis such as time to requirement of treatment (treatment free survival [TFS]) or overall survival [OS]). For these measures to reliably determine the effect of the marker, such as an *ATM* mutation, requires that the marker is present at the time of diagnosis. As I concluded in the last chapter, the majority of *ATM* mutations in CLL tumours were acquired within the tumour

cells and only a minority represented germ-line mutations. The timing of the acquired *ATM* mutations relative to the disease course is therefore important when assessing their potential impact on clinical outcome in CLL patients.

In this chapter, therefore, I have investigated the clinical effects of the *ATM* mutations in CLL patients. Initially, I have evaluated the impact of *ATM* mutations by assessing TFS and OS in the 155 patients from the unselected cohort and, subsequently, I have investigated the associations of *ATM* mutations with other prognostic parameters.

In addition, I investigated the clinical outcome in the cohort of 53 patients that all had a deletion of chromosome 11q. Here, I aimed to address the specific question, as to whether the addition of a mutation in the remaining *ATM* allele could further impact on outcome in this subgroup of CLL patients that already have a poor outlook.

Finally, I have studied the timing of the *ATM* mutations in relation to CLL ontology to determine both how this impacts on measurements of disease outcome and also to gain further insight into disease biology in order to assess how this may affect future treatment strategies.

5.2 Nature and clinical characteristics of the unselected CLL cohort

The nature of the unselected CLL cohort and the way that it was established is described in results chapter one. Briefly, patients were included on the basis of having a diagnosis of CLL and attending specific outpatient clinics over a defined study period. This led to a cohort where patients had originally been diagnosed with their disease over a considerable range of

time. From the cohort of 155 patients, 49 patients were diagnosed prior to 1996, 54 patients between 1997 and 2000, and 51 patients between 2001 and 2003. The assessment of disease outcome was based on survival and treatment status on December 1st 2004. Therefore, this represented a retrospective study with outcome measures taken from diagnosis. Due to the variation in the time of diagnosis, there was consequently considerable variation in the length of follow up, which ranged from a minimum of 25 months to a maximum of 217 months. The median follow up from diagnosis was 71 months.

The cohort of 155 patients consisted of 91 men and 64 women and the age ranged from 28 to 92 years, with a median age of 67 years. At diagnosis, 120 patients had Binet stage A disease, and 31 patients had more advanced stage disease (Binet stages B or C). The *IGVH* mutation status had been determined on the majority of the patients that formed this cohort. Forty-one patients were found to have unmutated *IGVH* genes and 97 patients to have mutated *IGVH* genes. Unfortunately, FISH data was not available on all patients but either karyotypic or FISH data was obtained on 77 patients from this cohort and 9 patients (12%) were known to have a chromosome 11q deletion. At the time of data analysis, 71 patients had received treatment for their disease and 34 patients had died (Table 5.1).

5.3 Impairment of clinical outcome in patients with *ATM* mutations

Table 5.1 – Clinical characteristics of the cohort of 155 CLL patients

Parameter	155 CLL cohort
Male : Female	91:64
Median age at Diagnosis (years)	67
Age range at Diagnosis (years)	28 - 92
Stage at diagnosis A B or C	(n=151) 120 31
Stage at genetic analysis A B or C	(n=147) 73 74
IGVH mutation status Unmutated Mutated	(n=138) 41 97
Chromosome 11q status Deletion No deletion	(n=77) 9 68
Era of diagnosis Pre 1996 1997-2000 2001-2003	 49 54 51
Median follow up (months)	71
Range of follow up (months)	25 - 217
Number of patients treated (December 2004)	(n=152) 71
Percentage treated	47
Median time to treatment (months)	90
Number of patients died (December 2004)	(n=154) 34
Percentage died (%)	22
Median Survival (months)	>220

In order to investigate the clinical impact of *ATM* mutations, I compared overall survival and treatment free survival between patients with *ATM* mutations and those that had wild type *ATM*.

Altogether, 34 out of 155 CLL patients had died at the time of the data analysis on December 1st, 2004. Amongst the patients with wild type *ATM*, 26/137 individuals had died, representing 19%, and amongst patients with *ATM* mutations 8/17 individuals had died, representing 47% (Table 5.2). Due to the variation in the length of follow up between patients, it was necessary to plot Kaplan Meier survival curves and use the log rank test to determine if there was a significant difference in survival outcome according to *ATM* mutation status. Using these methods, I was able to confirm that patients with *ATM* mutations had a significantly shorter survival from diagnosis compared to patients with wild type *ATM* (p=0.0034) (Figure 5.1).

In addition, I was able to show that patients with an *ATM* mutation in their tumour cells had a statistically significant reduction in treatment free survival, p=0.007, compared to patients whose tumours retained a wild type *ATM* gene (Figure 5.2). Altogether, 71 of 152 CLL patients from the cohort (47%) had received treatment for their disease at the time of data analysis and amongst the patients with wild type *ATM* 59/135 (44%) patients had been treated compared to 12/17 (71%) of patients with *ATM* mutations (Table 5.2).

5.4 Relationship between *ATM* mutations and other biomarkers

I reasoned that the poorer overall and treatment free survival observed in the patients with *ATM* mutations could be due to a positive association between *ATM* mutations with other prognostic factors including age, gender,

Table 5.2 – Clinical characteristics of the cohort of 155 CLL patients according to the mutation status of the *ATM* gene

Parameter	Wild type (n=137)	*ATM* mutant (n=18)
Male : Female (M/F ratio)	81:56	10:8
Median age at diagnosis (years)	66	68
Stage at Diagnosis A B or C	(n=134) 107 27	(n=17) 13 4
Stage at genetic analysis A B or C	(n=130) 66 64	(n=17) 7 10
IGVH gene Unmutated Mutated	(n=120) 31 89	(n=18) 10 8
11q deletion (n=77) present absent	(n=60) 5 55	(n=17) 4 13
TP53 status Mutant Wildtype	(n=137) 4 133	(n=18) 2 16
Era of diagnosis Pre-1996 1997-2000 2001-2003	 44 47 46	 5 7 5
Median follow up (months)	68.5	79
Range of follow up (months)	25-217	26-156
Number of patients treated	(n=135) 59	(n=17) 12
Percentage treated (%)	44	71
Median time to treatment (months) (n=152)	130 (n=135)	40 (n=17)
Number of patients died	(n=137) 26	(n=17) 8
Percentage died (%)	19	47
Median Survival (months) (n=154)	>217 (n=137)	85 (n=17)

Figure 5.1

There is reduced overall survival of CLL patients whose tumour cells have *ATM* mutations in comparison to those with wild type *ATM*. This difference is statistically significant (p=0.0034).

Figure 5.1

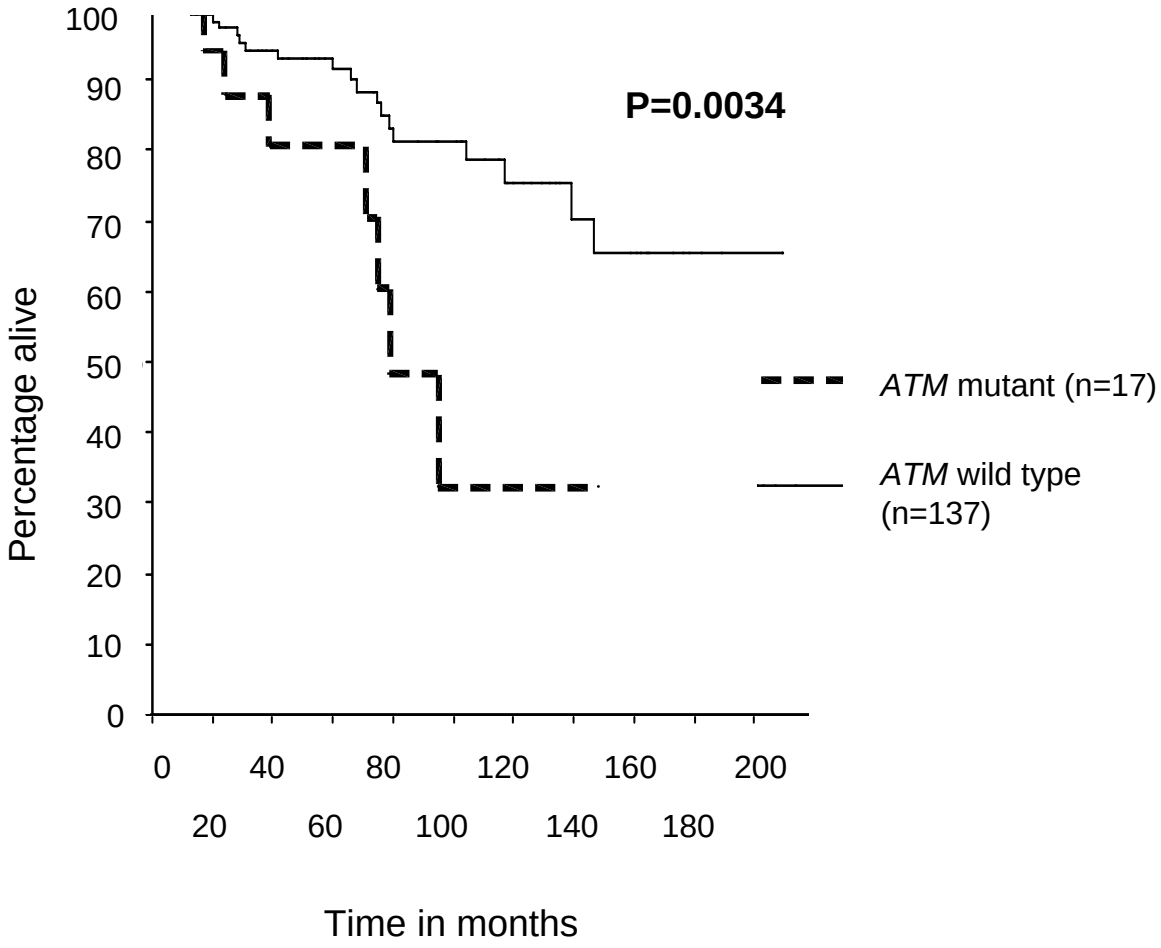
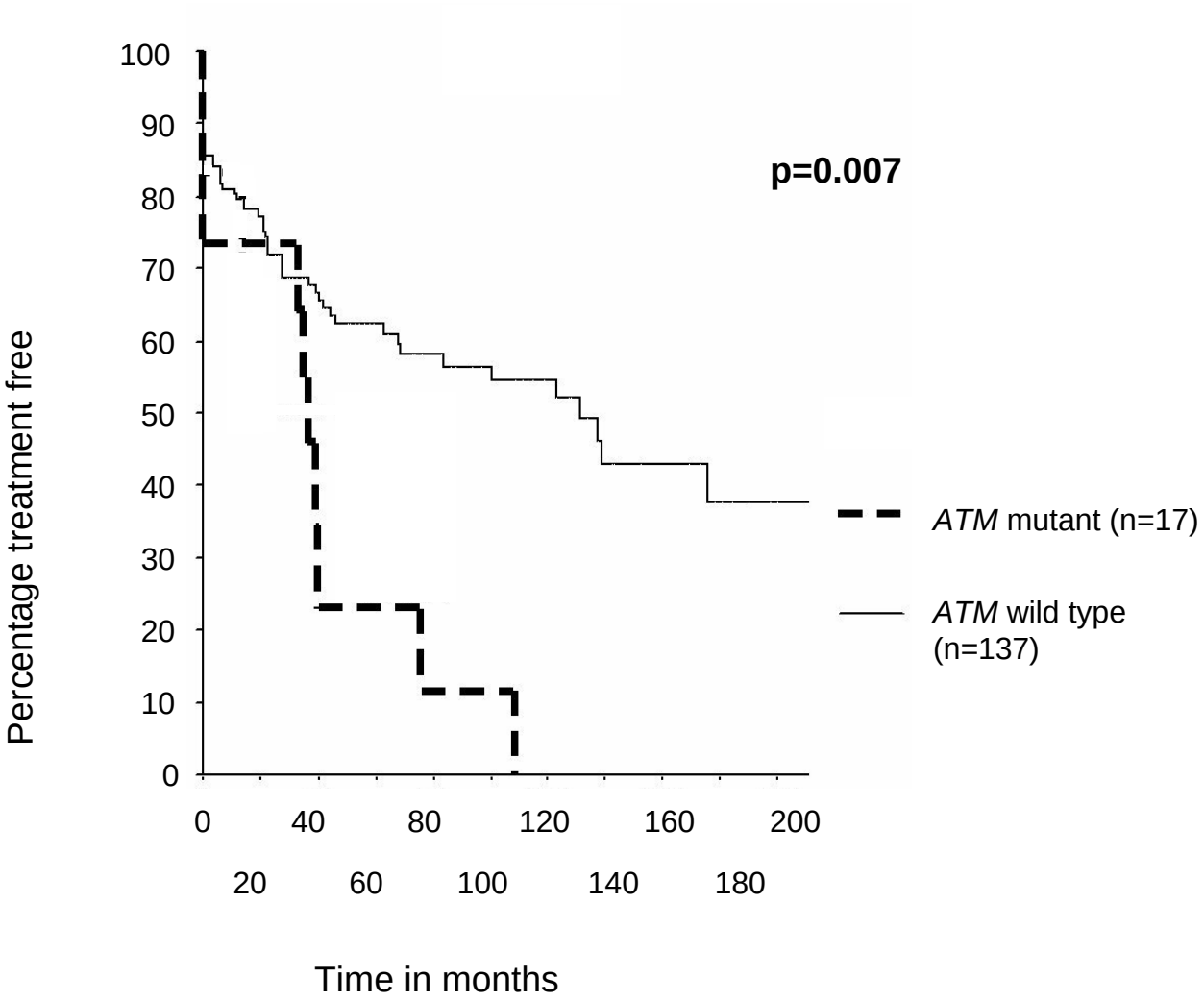

100
90
80
70
60
50
40
30
20
10
0
Percentage alive
P=0.0034
0
40
80
120
160
200
20
60
100
140
180
Time in months
ATM mutant (n=17)
ATM wild type (n=137)

Figure 5.2

There is significantly reduced treatment free survival of CLL patients whose tumour cells have *ATM* mutation in comparison to those with wild type *ATM* (p=0.007).

Figure 5.2


100
90
80
70
60
50
40
30
20
10
0
Percentage treatment free
p=0.007
ATM mutant (n=17)
ATM wild type (n=137)
0
40
80
120
160
200
20
60
100
140
180
Time in months

stage, *IGVH* mutation status, *TP53* mutation status and cytogenetic abnormalities. Increased age, male sex, advanced clinical stage, unmutated *IGVH* genes and *TP53* mutations are all known to be associated with a poorer outcome in CLL patients (Catovsky D *et al*, 1989, Chiorazzi N *et al*, 2005, Sellick GS et al, 2006, Hamblin T *et al*, 1998, Dohner H *et al*, 1999). The cytogenetic changes that are associated with poor clinical outcome are deletions of chromosome 17p, which lead to the loss of the *TP53* gene and are associated with tumours with *TP53* mutations, and deletions of 11q that lead to the loss of one *ATM* allele. The relationship between *ATM* mutations and these prognostic factors in the cohort of 155 unselected patients is considered below and summarised in Tables 5.2 and 5.3.

5.4.1 Age and Gender

There was no difference in the median age at diagnosis between patients with *ATM* mutations (68 years) and those with wild type *ATM* genes (66 years). The male to female ratio (M:F) in the CLL patients whose tumours had *ATM* wild type genes was approximately 7:5, and a slightly higher proportion of women was noted amongst the *ATM* mutant CLLs (M:F 5:4). Since women are known to have a better outcome then men in CLL, the higher proportion of women in the *ATM* mutant group would be expected, if anything, to reduce the negative impact of an *ATM* mutation.

5.4.2 Disease Stage

There was no significant difference in the proportion of patients with limited stage disease (Binet stage A) versus those with advanced stage disease

Table 5.3 – Clinical parameters in patients with an *ATM* mutation from the 155 unselected cohort

Patient	Sequence change	Protein change	Age at diagnosis	Stage at diagnosis	*IGVH* mutation status and family	OS mths	TFS mths
CLL77	1058DelGT 5224G/C	Truncated protein A1742P	62	B	99%, VH1-2,	83+	0
CLL96	2929Ins9 5041A/G 5044G/T	Inframe insertion I1681V D1682Y	75	A	96%, VH4-61	79	33
CLL11	7313C/A 8833Del34	T2438K Truncated protein	59	A	98%, VH3-15	156+	108
CLL57*	2308G/T	Truncated protein	68	A	97%, VH4-59	101+	76
CLL15*	7047C/G	C2349W	61	A	100%, VH1-69	75	40
CLL07*	8600G/A	G2867E	74	A	99%, VH3-11	96	0
CLL152*	8839A/T	T2946S	77	C	99%, VH1-6	79+	35
CLL75	1009C/A	R337S	52	B	98%, VH3-30	39	0
CLL69	6815DelA	Truncated protein	67	A	100%, VH3-15	72+	39
CLL119	9022C/T	R3008C	62	A	97%, VH3-64	47+	36
CLL93	5290del C	Truncated protein	69	A	100%, VH5-51	26+	26+
CLL92	5980A/G	K1994E	86	A	99%, VH1-69	24	24+
CLL124[1]	5228C/T	T1743I	85	A	85%, VH3-15	48+	48+
CLL84[1]	8592C/T	Splicing defect	89	A	93%, VH3-7	17	14
CLL27	3383A/G	Q1128R	74	C	99%, VH3-21	77	0
CLL113	3964C/A	L1322F	68	A	91%, VH3-7	24	24+
CLL147	IVS16-10T/G	Exon skip	nk	nk	94%, VH5-51	NA	NA
CLL81	5882A/G	Y1961C	65	A	93%, VH3-15	36+	36+

* Denotes tumours with an 11q deletion, [1] Denotes tumours with a *TP53* mutation

nk - not known, + either still alive or untreated

(Binet stages B and C) at the time of diagnosis between patients with *ATM* mutations (13 vs 4) and patients with wild type *ATM* genes (107 vs 27). At the time of genetic analysis, there was a slightly higher proportion of patients with *ATM* mutations with advanced stage versus limited stage disease (10 vs 7) compared to patients with wild type *ATM* genes (64 vs 66) however these differences remained non-significant. The date of the genetic analysis was an arbitrary one with respect to the course of any individual's disease but occurred some time after the diagnosis for the majority of cases.

5.4.3 *IGVH* mutation status

In contrast to age and stage, there *was* a significant association between *ATM* mutations and *IGVH* mutation status. In this study tumours were classified as having unmutated *IGVH* genes if there was 98% or greater concordance with the germ-line sequence and having mutated *IGVH* genes if there was less than 98% concordance with the germline sequence. Altogether, the *IGVH* mutation status was known on 138 patients and 41 (30%) had unmutated *IGVH* genes. There was a higher proportion of tumours with unmutated *IGVH* genes among the subgroup of *ATM* mutant tumours 56% (10/18) compared to either the *ATM* wild type tumours 26% (31/120). Therefore 10 of the 41 tumours with *IGVH* unmutated genes had *ATM* mutations (24%), compared to 8 of the 97 tumours with *IGVH* mutated tumours that had an *ATM* mutation (8%). Using Fisher's exact test it was possible to show that there was statistically significant positive association between the tumours with unmutated *IGVH* genes and those with *ATM* mutations ($p=0.024$).

5.4.3.1 *ATM* mutations and *IGVH* status in relation to clinical outcome

It was possible, therefore, that the poorer overall and treatment free survival in the genetic subgroup of patients with *ATM* mutations was due to the positive association of *ATM* mutations with unmutated *IGVH* genes, rather than resulting from the biological consequences of the *ATM* mutations themselves. To investigate this possibility the survival data was subjected to further analysis.

Firstly, the prognostic value of the mutation status of the *IGVH* genes was analysed in this series. As expected, regardless of the *ATM* mutation status, the TFS survival was significantly shorter in patients with *IGVH* un-mutated than in patients with *IGVH* mutated genes ($p<0.0001$). Surprisingly, however, the difference in OS between the *IGVH* unmutated and mutated subgroups did not reach significance in this series although, nevertheless, there was a trend for poorer OS survival in patients with unmutated *IGVH* genes ($p<0.19$). Interestingly, the 10-year OS for mutated *IGVH* patients in our cohort was shorter (72%, vs 85%) compared to a previously published report (Hamblin T *et al*, 1999). In contrast, there was no difference in the 10-year OS for the patients with unmutated *IGVH* genes between this series and the previously published one. Thus the failure of *IGVH* gene mutation status to significantly predict OS in my series appeared to be due to an excess of deaths in the group with mutated *IGVH* genes rather than a decreased death rate in those with unmutated *IGVH* genes. This could be reflection of the fact that the mean and median ages in my cohort were slightly higher than in the previous report. In support of this idea, the subgroup analysis of only younger patients (<65

years) in this study demonstrated that *IGVH* mutation status was significantly predictive of OS (p=0.034).

Multivariate analysis using Cox regression was then performed on the 135 patients where information on age, disease stage, *IGVH* mutation status and *ATM* mutation status, was available. After adjustment for age, stage and *IGVH* mutation status, *ATM* mutations were still significantly predictive of OS (p=0.0025). The adjusted relative hazard (risk of death) for patients with *ATM* mutations, as compared to wild type, was 3.0 (95% confidence interval 1.23-7.29). In other words, patients with *ATM* mutations had a 3 fold higher risk of death at any point during their disease course than patients without *ATM* mutations even after adjusting for age, stage and *IGVH* mutation status. This confirmed that *ATM* mutations are an independent predictor of OS in CLL patients, with respect to these parameters.

Multivariate analysis was also used to assess if *ATM* mutations were an independent predictor of TFS. Since advanced stage is a specified criterion for treatment in CLL, clinical stage was not included in the multivariate analysis for TFS (Oscier D *et al*, 2004). By this method, it was possible to show that even after adjustment for age and *IGVH* status, *ATM* mutations were still significantly predictive of a shortened TFS (p=0.035). The relative hazard (risk of commencing treatment) for patients with *ATM* mutations, compared to wild type, was 2.44 (95% confidence interval 1.07-5.60). Thus, patients with *ATM* mutations were 2.44 times more likely to receive treatment than patients without *ATM* mutations even after adjustment for age and *IGVH* mutation status. This confirmed that *ATM* mutations are also an independent predictor of TFS in CLL patients with respect to age and *IGVH* status.

5.4.4 *TP53* mutation status

Abnormalities in the *TP53* gene that encodes the pro-apoptotic protein p53 are also known to occur in CLL tumours and have been shown to be associated with a poor prognosis and treatment resistance in CLL patients. Previously, *ATM* mutations and *TP53* mutations have occurred in distinct CLL tumour subgroups suggesting that they are identifying independent subgroups in CLL patients. In this project, I also screened the DNA from the 155 tumours for mutations in the *TP53* gene and subsequently compared the outcome in the three genetic subgroups.

I found six *TP53* mutations from the cohort of 155 tumours, which represented a prevalence of 4% (Table 5.4). Interestingly, I found that two of the tumours with a *TP53* mutation (CLL 84 and 124) also had a heterozygous *ATM* mutation (Tables 5.3 and 5.4). This combination of genetic defects has not been previously described in CLL tumours. Altogether, from the 155 tumours, there were therefore 16 tumours with an *ATM* mutation and wild type *TP53*, 4 tumours with a *TP53* mutation and wild type *ATM* and 2 tumours with a mutation in both the *ATM* and *TP53* genes. The prevalence of *ATM* mutations and *TP53* mutations in the cohort of 155 patients was 12% and 4%, respectively.

I next compared the OS and TFS between the three genetic subgroups, namely the *ATM* mutant, *TP53* mutant and *ATM/TP53* wild type. Since *TP53* mutations are known to have a detrimental effect on survival, I categorised the 2 tumours with an *ATM* and a *TP53* mutation into the group of *TP53* mutant

Table 5.4 - *TP53* mutations in the cohort of 155 CLL patients

Patient	Sequence change	Protein change
CLL29	807ins12	Inframe insertion
CLL84[1]	797G/A	G266Q
CLL110	536A	H179L
CLL124[1]	403T/C	C135R
CLL70	739A/T	N247Y
CLL146	455C/T	P152L

[1] Denotes tumours with an *ATM* mutation – CLL84 *(ATM* mutation 8592C/T) and CLL124 (*ATM* mutation 5228C/T)

tumours when making a comparison of the three genetic subgroups thereby preventing any bias towards adverse outcome in the *ATM* mutant group.

The difference in overall survival between all three genetic subgroups was also highly statistically significant ($p<0.0001$). The patients whose tumours carried *TP53* mutations had the shortest OS, and the patients with *ATM* mutations had an OS that was intermediate with the two other genetic subgroups. The median OS was 85 months for patients with an *ATM* mutation, 44 months for patients with *TP53* mutations and was not reached at greater than 12 years for patients with *ATM/TP53* wild-type tumours (Figure 5.3). There was also highly statistically significant difference in treatment free survival ($p=0.00153$) between the three genetic groups (Figure 5.4). The median TFS was 40 months for both patients with an *ATM* mutation and patients with a *TP53* mutation in their tumour cells compared to 130 months for patients that had wild type *ATM/TP53* genes.

These results show that *ATM* mutations have an adverse effect on survival in CLL patients, which is less severe than the effect of *TP53* mutations, and is also independent of *TP53* mutations.

5.4.5 Chromosome 11q deletion

CLL patients with a chromosome 11q deletion have a poor clinical outcome. By definition, these patients have loss of one *ATM* allele because it is an ATM probe that is used for the detection of the deletion. Therefore, I was interested to investigate the relationship between *ATM* mutations and 11q deletions in CLL patients, in terms of clinical parameters. From the cohort of 155 patients, there was information on cytogenetic abnormalities on 77

Figure 5.3

The overall survival of patients with *ATM* mutations was intermediate between the survival of patients with *TP53* mutations and the survival of patients with wild type *ATM* and *TP53* genes. In this analysis the two patients with a mutation in both *TP53* and *ATM* genes were included in the category of patients with *TP53* mutations. Therefore the poor outlook of the patients with *ATM* mutations was independent of *TP53* mutations in this cohort. The differences between all three genetic subgroups were highly statistically significant ($p<0.001$).

Figure 5.3

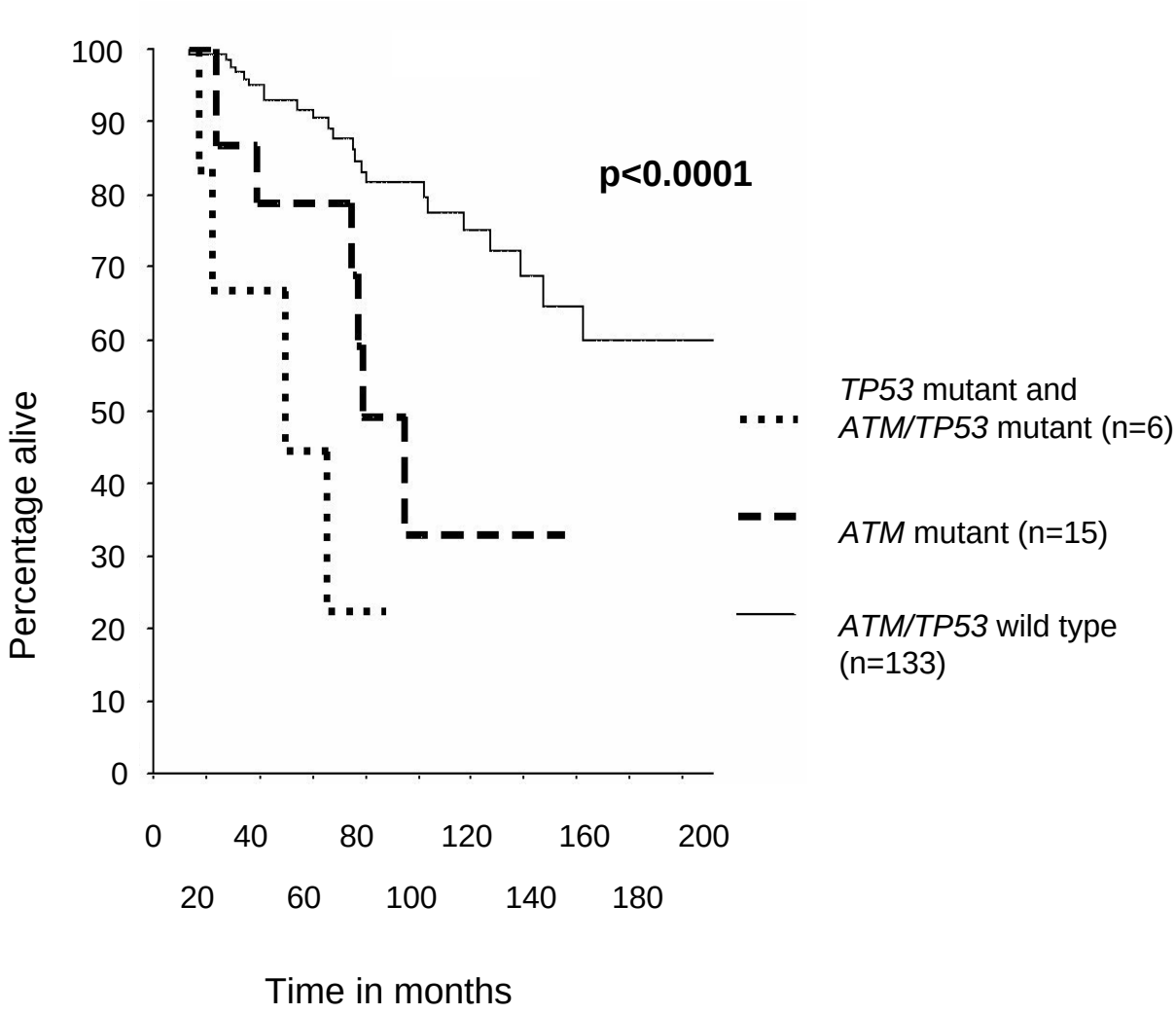
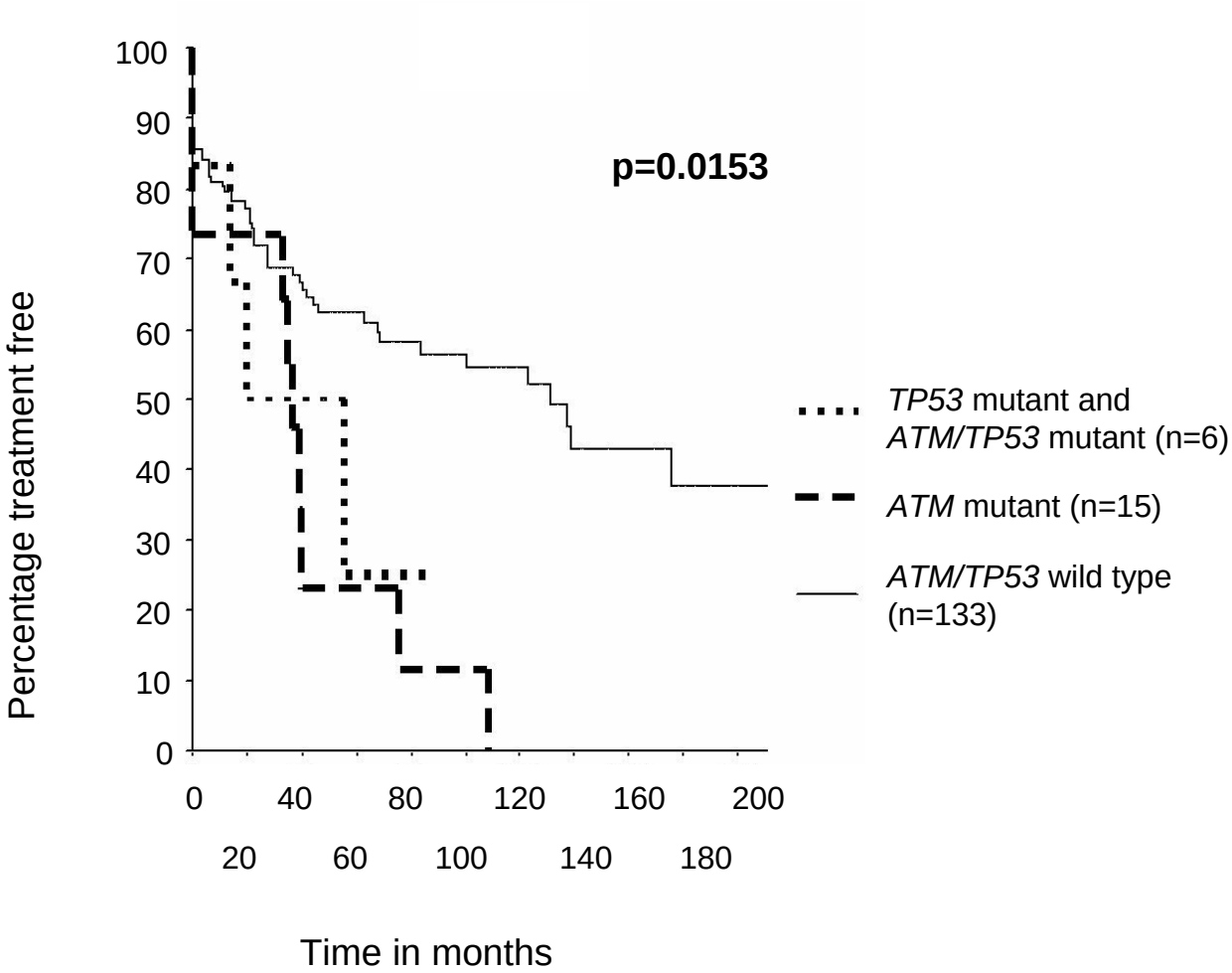

100
90
80
70
60
50
40
30
20
10
0
Percentage alive
p<0.0001
0
40
80
120
160
200
20
60
100
140
180
Time in months
TP53 mutant and ATM/TP53 mutant (n=6)
ATM mutant (n=15)
ATM/TP53 wild type (n=133)

Figure 5.4

The effects of *ATM* mutations on treatment free survival were also independent of the presence of *TP53* mutations. Both patients with *ATM* mutations and those with *TP53* mutations had a significantly shorter treatment free survival than those patients with wild type *ATM* and *TP53* genes.

Figure 5.4


100
90
80
70
60
50
40
30
20
10
0
Percentage treatment free
p=0.0153
0
40
80
120
160
200
20
60
100
140
180
Time in months
TP53 mutant and *ATM/TP53* mutant (n=6)
ATM mutant (n=15)
ATM/TP53 wild type (n=133)

tumours and there were 9 tumours that were known to have a deletion of chromosome 11q. Four of these nine tumours (44%) also had an *ATM* mutation, and 5 (56%) had wild type *ATM*. FISH analysis was available on 17 patients with an *ATM* mutation and therefore 24% (4/17) also had a deletion of chromosome 11q. These results indicate that there is only a partial correlation between tumours with *ATM* mutations and those with an 11q deletion in this cohort. However, the 11q status was not known on all the patients that had wild type *ATM* in this study. Therefore in order to investigate the relationship between these two prognostic markers, I established the second cohort of 53 tumours that all had an 11q deletion.

This selected cohort of 53 patients with an 11q deletion consisted of 31 men and 22 women. The median age at diagnosis was of 64 years and ranged from 24 to 90 years. The median follow-up from diagnosis was 78.5 months and ranged from 12 to 324 months. The clinical stage at diagnosis was known for 47 patients, and 32 patients (68%) had limited stage A disease compared to 15 patients (32%) with advanced stage disease (B and C). The immunoglobulin *VH* status was known in 38 patients and 28 (74%) had un-mutated *VH* genes (98% or greater concordance with the germ-line sequence) whereas 10 (26%) had mutated *VH* genes (less than 98% concordance with the germ-line sequence) The clinical characteristics of this total cohort and the two subgroups according to the status of the remaining *ATM* allele are summarised in Tables 5.5, 5.6 and 5.7. There were no significant differences in parameters such as age, clinical stage or *IGVH* mutation status between the two subgroups.

Table 5.5 – Clinical characteristics of 53 CLL patients with an 11q deletion

Parameter	**Total cohort (n=53)**	**Subgroup with a mutant *ATM* allele (n=20)**	**Subgroup with a wild type *ATM* allele (n=33)**
Male : Female	31:22	11:9	20:13
Median age at Diagnosis (years)	64	68	61
Age range	24-90	49-90	24-91
Stage at diagnosis A B or C	(n=47) 32 15	(n=18) 12 6	(n=29) 20 9
IGVH mutation status Unmutated Mutated	(n=38) 28 10	(n=15) 12 3	(n=23) 16 7
Range of follow up (months)	12 - 324	15-273	12-324
Number of patients treated	(n=51) 39	(n=19) 15	(n=32) 24
Percentage treated	76	79	75
Median time to treatment (months)	30	22	32
Number of patients died	(n=51) 28	(n=19) 12	(n=32) 16
Percentage died	55	63	50
Median Survival (months)	88	83	117

Table 5.6 - Clinical parameters in patients with an 11q deletion and an *ATM* mutation

Patient	Nucleotide Change	Protein Change	Age at diagnosis	Stage at diagnosis	IGVH status	% of cells with 11q deletion	Overall survival (months)
CLL189	9023G/A	R3008H	67	A	UM	88	17+
CLL166	8977C/T	R2993X	51	B	nk	99	56
CLL174	8861A/G	Y2954C	86	A	M	54	43
CLL152	8839A/T	T2947S	77	C	UM	99	82
CLL192	IVS62-1G/T	Splice defect	63	A	UM	43	30+
CLL07	8600G/A	G2867E	74	A	UM	92	96
CLL200	8249T/C	L2750S	71	A	UM	86	55
CLL182	8249T/C	L2750S	49	nk	nk	69	17+
CLL193	8056T/C	F2686L	90	A	UM	nk	21
CLL183	7883del5	Truncated	84	nk	nk	84	14+
CLL15	7047C/G	C2349W	61	A	UM	95	75
CLL162	6106T/A	Y2036N	68	A	M	86	46
CLL203	6067G/A	G2023R	52	C	UM	45	88
CLL169	5980A/G	K1994E	86	A	UM	100	30
CLL178	4591C/T	Q1531X	54	B	nk	nk	85+
CLL180	3883del3	Deletion L	nk	A	nk	96	nk
CLL198	3651delG	Truncated	49	C	UM	nk	69+
CLL173	2417T/G	L806W	54	A	UM	26	86
CLL57	2308G/T	E770X	68	A	M	90	110+
CLL170	1120C/T	Q374X	79	B	UM	nk	128

UM unmutated; M mutated; ND not done; nk not known; + still alive

Table 5.7 - Clinical parameters in patients with an 11q deletion and no *ATM* mutation

Patient	Age at Diagnosis	Stage at diagnosis	IGVH status	% cells with 11q deletion	Overall survival (months)
CLL160	56	A	M	34	78
CLL161	48	A	UM	95	32+
CLL163	79	A	UM	87	117
CLL164	49	nk	NK	47	14+
CLL165	39	A	UM	95	163
CLL19	61	C	UM	95	34
CLL167	nk	nk	nk	47	nk
CLL168	82	A	nk	41	116+
CLL25	49	B	UM	97	50+
CLL171	83	nk	nk	10	12+
CLL37	47	B	UM	75	44+
CLL172	77	B	UM	40	65
CLL175	76	nk	nk	11	6
CLL176	79	A	UM	nk	134
CLL177	77	A	UM	74	45
CLL179	68	A	nk	nk	275
CLL185	42	A	nk	90	25+
CLL186	24	A	UM	nk	149
CLL187	72	B	M	21	169
CLL65	91	A	M	91	28
CLL188	42	A	M	7	124+
CLL190	48	B	nk	50	25+
CLL71	64	A	UM	60	36+
CLL191	56	C	nk	94	32+
CLL194	52	A	nk	23	65+
CLL195	73	B	UM	nk	26
CLL196	52	A	M	44	322+
CLL197	76	A	UM	nk	117
CLL199	70	A	M	22	49
CLL201	75	A	M	nk	46
CLL138	34	C	UM	80	128+
CLL202	28	A	UM	nk	27+
CLL204	61	A	UM	nk	4+

UM unmutated; M mutated; ND not done; nk not known; + still alive

In this second cohort of patients with an 11q deletion there were a higher number of patients that had poor prognostic disease features compared to the unselected cohort, irrespective of *ATM* mutation status. In the 11q-deleted cohort, 74% of patients had un-mutated *IGVH* genes compared to only 30% of patients in the unselected cohort. There were also more patients that had advanced stage disease at diagnosis in the 11q-deleted cohort compared to the unselected cohort, 32% vs 21%, respectively. However there was no difference in the ages at diagnosis (median 64 years for the selected cohort vs 67 years for the unselected cohort) or gender distribution (male:female 1.41:1 for the selected cohort vs 1.42:1 for the unselected cohort) between the two cohorts (Tables 5.1 and 5.5).

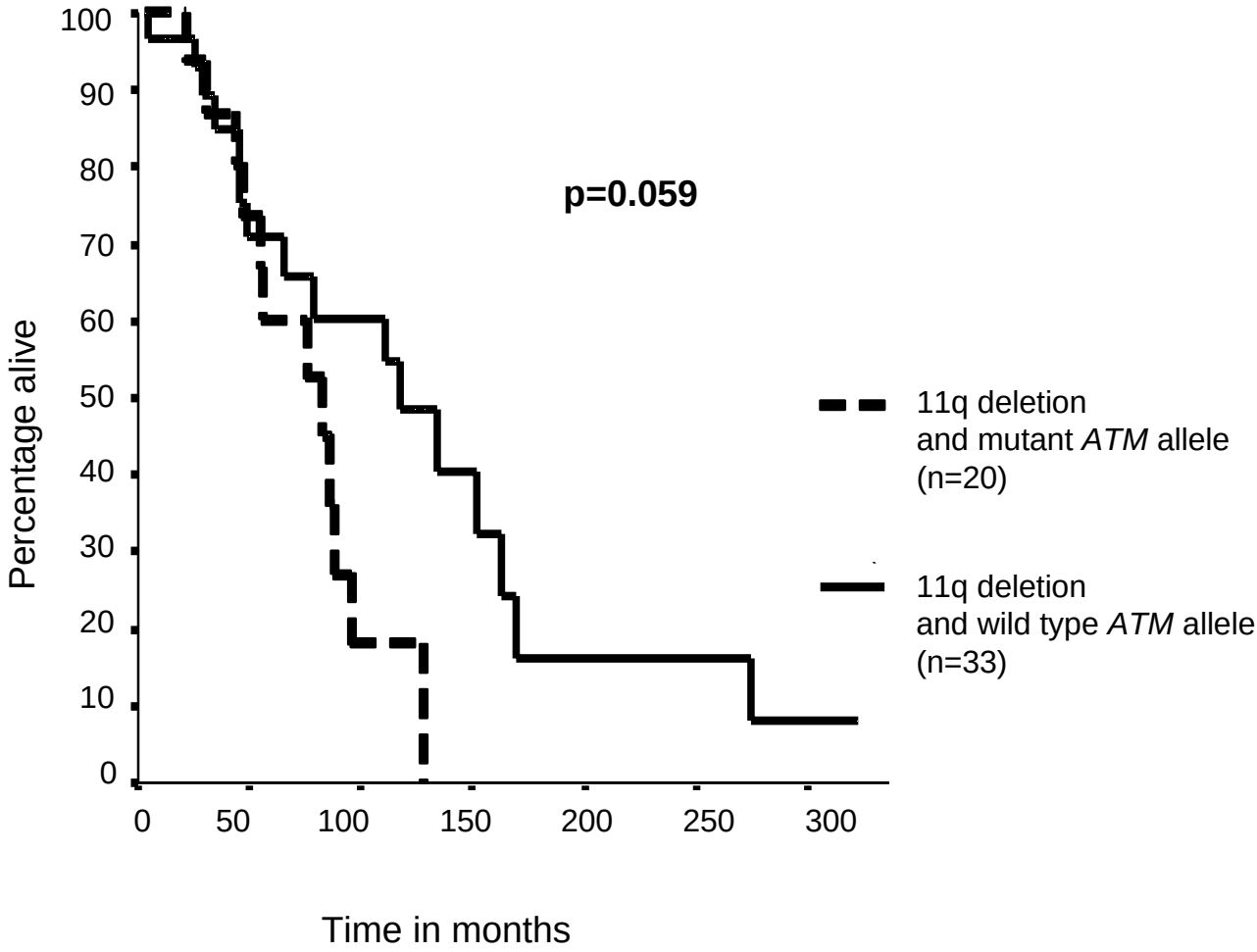
Altogether from this cohort of tumours with an 11q deletion, 39 out of 51 (76%) patients had been treated and 28 out of 51 (55%) patients had died at the time of the data analysis on 01.09.05. The median overall survival was 88 months and the median treatment free survival was 30 months. These were both markedly shorter than within the unselected cohort where the median overall survival had not been reached at 220 months of follow up and where the median treatment free survival was 90 months. Thus the poor prognosis of a chromosome 11q deletion was confirmed in this study.

I next compared clinical parameters between the two subgroups of 11q-deleted tumours, those with a second mutant *ATM* allele and those with a second wild type *ATM* allele. Interestingly, there was a trend for a shorter overall survival, which approached significance, for the group of patients with an 11q deletion and an additional mutant *ATM* allele compared to the group with a second wild type *ATM* allele (p=0.059) (Figure 5.5). The median overall

Figure 5.5

There was a trend for a poorer overall survival amongst patients with an *ATM* mutation in addition to an 11q deletion in comparison to patients with an 11q deletion that had a second wild type *ATM* allele. However the differences in survival for this cohort of 53 patients did not reach statistical significance (p=0.059).

Figure 5.5


100
90
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70
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50
40
30
20
10
0
Percentage alive
0
50
100
150
200
250
300
Time in months
p=0.059
11q deletion
and mutant *ATM* allele
(n=20)
11q deletion
and wild type *ATM* allele
(n=33)

survival was 83 months (95% confidence interval (CI) 50.2 -115.9) in patients with a mutant *ATM* allele compared to 117 months (95% CI 54.6 – 179.8) in the group with a wild type *ATM* allele. Moreover, no patients with an *ATM* mutation survived beyond 128 months. The ten-year survival within this group was just 18% (95% CI 0% – 40.1%) compared to 54% (95% CI 33.3% - 76.3%) in patients with a wild type *ATM* allele.

I subsequently compared the treatment free survival between the two groups. There was no significant difference between the subgroups for this parameter (p=0.24) suggesting that the trend for a shorter overall survival in the subgroup of patients with an additional *ATM* mutation was due to the combination of a slightly shorter TFS but also a shorter survival post treatment with chemotherapy.

5.5 Effect of a retrospective cohort

The ideal method to examine the prognostic significance of a biological marker, such as the mutation status of the *ATM* gene, would be to analyse each tumour sample at diagnosis and then follow up patient outcome prospectively. Unfortunately, since the median survival in CLL is greater than 10 years, such a study would take many years and, therefore, all published studies investigating potential prognostic markers in CLL have taken a retrospective approach (Hamblin T *et al*, 1999, Dohner H *et al*, 2000, Wiestner A *et al*, 2003). However, there are disadvantages to retrospective studies. For example, if a biological marker has a detrimental effect on clinical outcome, then more patients with this marker in their tumour cells will have died *before*

the time of the analysis, than those without this marker, and therefore the biological marker's prognostic value could be underestimated.

In order to investigate this possibility in the unselected cohort of 155 patients, I divided the patients into three groups according to the 'era of diagnosis' and included this in multivariate analysis. The first era included those that were diagnosed before December 31st 1996, the second those that were diagnosed between the years 1997 and 2000 inclusively, and the third era those that were diagnosed years between the years 2001 and 2003 inclusively (Table 5.3). After the inclusion of era of diagnosis, the hazard ratio (risk of death) for *ATM* mutations increased from 3.0 to 3.4, indicating that in this retrospective type of study, the effect of *ATM* mutations on OS is actually an underestimation.

5.6 Timing of *ATM* mutations in the pathogenesis of CLL

The time at which an *ATM* mutation develops is important when assessing the impact of *ATM* mutations on clinical outcome and evaluating their potential role as a prognostic biomarker. If an *ATM* mutation is acquired some time during disease progression after the initial disease diagnosis, then any potential adverse effect of this mutation will be underestimated when using clinical outcome parameters that are measured from the time of diagnosis. Nevertheless, differences in clinical outcome might still be evident if the mutations lead to a marked effect on tumour cell behaviour.

I evaluated the timing of the *ATM* mutations in selected patients where tumour samples from more than one time point during the disease course were available. Altogether, there were seven tumours (CLLs 77, 96, 07, 75,

152, 173 and 198) in which I had detected an *ATM* mutation where tumour material was available from a time-point earlier in the disease course and could therefore be examined for the presence of the same mutation as part of a 'look back' analysis (Table 5.8). In five cases (CLLs 77, 96, 07, 75 and 152), the *ATM* mutations were also detected in tumour material collected at an earlier date. For example in CLL 77, the mutation 5224G/C was present at diagnosis in 1998 as well as in 2002 and additionally this tumour also had the germ-line mutation 1058delGT indicating that both *ATM* mutations were present at the time the CLL was diagnosed. In the case of CLL96 the three mutations 2929ins 9, 5041A/G and 5044G/T were all present at diagnosis in 1996 and as well as in 2002. In, CLL 07 the mutation 8600G/A was detected in 1995 at diagnosis as well as in 2002 and this tumour also had loss of the second *ATM* allele through a chromosome 11q deletion. In, CLL 75 the mutation 1009C/A was found both in 2000 at diagnosis and in 2002, and finally, in CLL 152 the mutation 8839A/T was found both in 1999 (one year following the diagnosis of the disease) and in 2002.

In two cases (CLL173 and 198), the *ATM* mutations were not detected in tumour material collected at an earlier date as part of the 'look back' analysis. In CLL173, the mutation 2417T/G was detected in 2001 but was not observed in 1995 at the time of diagnosis of CLL. Similarly, for CLL198, the mutation 3651delG was detected in 2003 but was not present in 2001 at the time of diagnosis. Both these tumours also had loss of the second *ATM* allele through a chromosome 11q deletion (Figure 5.6).

Notably, all five tumours (CLLs 07, 75, 77, 96 and 152) analysed in this manner that originated from the unselected cohort had the *ATM* mutation

Table 5.8 - 'Look Back Analysis' to investigate the timing of the acquired *ATM* mutations

Patient	Date of diagnosis	Date of earliest sample Presence or Absence of mutation in this sample	Date of later sample Presence or Absence of mutation in this sample
CLL 07	01.01.95	01.01.95 8600G/A (A) (11q status - UK)	25.04.02 8600G/A (A) 11q deletion
CLL 75	02.02.00	23.02.00 1009C/A (A)	14.10.02 1009C/A (A)
CLL 77	14.01.98	14.01.98 5224G/C (A) 1058delGT (G)	17.12.02 5224G/C (A) 1058delGT(G)
CLL 96	11.11.96	1996 5041A/G (A) 5044G/T (A) 2929ins9 (A)	19.03.02 5041A/G (A) 5044G/T (A) 2929ins9 (A)
CLL 152	05.01.98	1999 8839A/T (A) (11q status - UK)	07.10.02 8839 A/T (A) 11q deletion
CLL 173	07.09.95	10.10.95 Not present (11q status - UK)	01.08.01 2417T/G (A) 11q deletion
CLL 198	01.01.00	26.03.01 Not present (11q status - UK)	05.02.03 3651delG (A) 11q deletion

A = Acquired *ATM* mutation, G= Germline *ATM* mutation

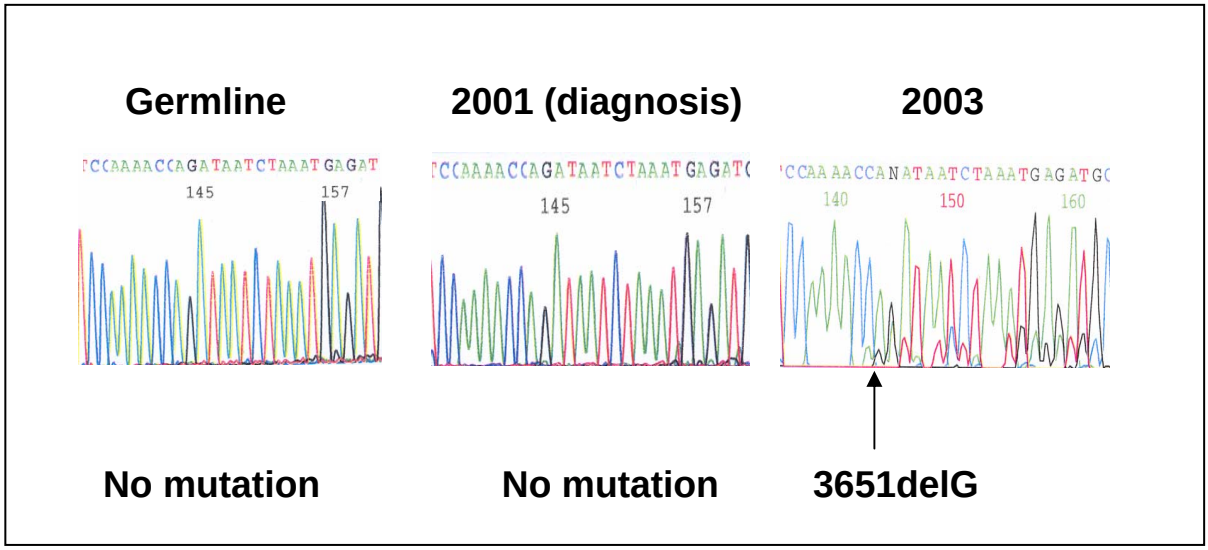
Uk - Unknown

Figure 5.6

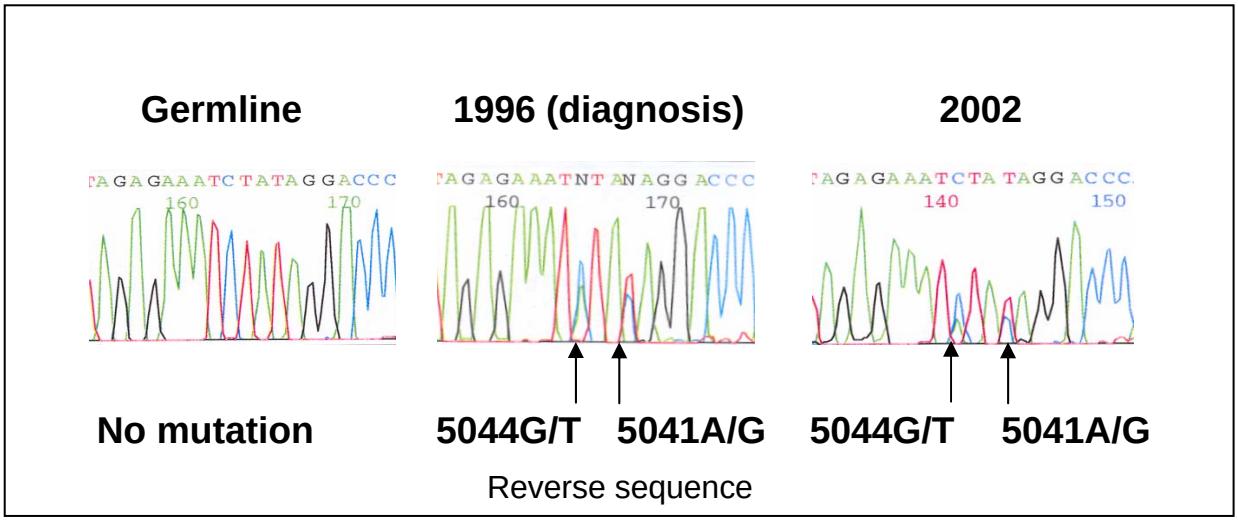
The timing of the development of ATM mutations varied in different CLL tumours. In CLL198 the *ATM* mutation, 3651delC, was present in the tumour sample analysed in 2003 but absent from both germ-line material and the diagnostic sample in 2001. By comparison the two mutations, 5041A/G and 5044G/T, in CLL 96 were also absent from the germ-line but they were present in diagnostic material from 1996. Thus acquired mutations in *ATM* can occur both early at disease onset and later during the progression of the CLL clone.

Figure 5.6

CLL198



CLL96



present in both the early and later sample. In a further three tumours (CLLs 92, 113 and 81) the *ATM* mutations that were detected were known to have been present in diagnostic material. Thus, altogether, from 22 mutations in the 18 patients in this cohort, there were ten mutations that were present within the tumour cells at the time of diagnosis. A further mutation was present in a sample collected at one year following diagnosis suggesting that it was also acquired early in the disease course. The timing of the remaining mutations was unknown but, nevertheless, these results indicate that *ATM* mutations are commonly early events in CLL.

Of the four tumours analysed by this analysis in the 11q-deleted cohort, two tumours (CLL 07 and 152) (these were common to both cohorts) had the mutation in both the early and later samples, whereas two tumours (CLLs 173 and 198) only had the mutation present in the later sample. In this cohort, in addition to CLLs 07 and 152, there were a further eight tumours where the mutations was known to be present within one year of diagnosis. Altogether, the results from the selected 11q-deleted cohort suggest that although *ATM* mutations are present at diagnosis in a proportion of tumours, in others they may be acquired during disease progression.

Interestingly, in the two cases, CLLs 173 and 198, where it was possible to definitively confirm the acquisition of an *ATM* mutation at a time after the diagnosis of CLL, the patients had both received treatment with a DNA damaging chemotherapeutic drug, chlorambucil, between the earlier and later samples tested for *ATM* mutations.

5.7 Conclusion

In order to be clinically useful as a prognostic marker, *ATM* mutations should impact on disease outcome, provide independent prognostic information and ideally should be present at the time of clonal transformation. Here I have shown that within unselected CLL tumours, *ATM* mutations significantly impact on both TFS and OS in a manner that is independent of age, clinical stage, *IGVH* mutation status and *TP53* mutation status.

I have also confirmed that there is an incomplete correlation between tumours with *ATM* mutations and those with 11q deletions in CLL. Therefore, this suggests that the detection of both patient subgroups would be complementary in the identification of patients that will have a poor clinical outcome. In this study there was also a trend, although this did not reach significance, which suggested that within the subgroup of patients with an 11q deletion, those patients that have an additional *ATM* mutation might have a poorer overall survival from diagnosis than the patients that retains one wild type copy of an *ATM* allele.

Finally, I have shown that *ATM* mutations are commonly early events in the pathogenesis of CLL suggesting that they would indeed have value as a prognostic marker. However in certain cases, perhaps particularly when they represent a second abnormality in the *ATM* gene they can occur after diagnosis and during disease progression. This suggests there maybe a step-wise development of abnormalities in the *ATM* gene in CLL.

Results 3

The effects of the *ATM* mutations on the DNA damage response

6.1 Introduction

In this part of my project I wanted to address the functional consequences of the *ATM* mutations which I had detected in the CLL tumour cells. One aim was to investigate potential assays that might allow the detection of those tumours that harbour *ATM* mutations via their functional responses. ATM coordinates an integrated cellular response to DNA DSBs and therefore it should be possible to study the effects of abnormal ATM activity at different levels in the DNA damage response pathway in CLL tumours (Kurz EU, Lees-Miller S, 2004). For example, following DNA damage, defects in ATM function might be expected to impact on the early ATM dependent phosphorylation of substrates, the later up-regulation of total p53 levels and its transcriptionally regulated substrates, and the subsequent induction of apoptosis via the p53-dependent intrinsic pathway. Since the phosphorylation of substrates is directly catalysed by ATM at early time points after the induction of DNA DSBs, this would be expected to most specifically reflect the ATM activity in the tumour cells. The assessment of the downstream consequences of the *ATM* mutations will also be important in order to validate their pathogenic nature and to determine their ultimate effect on cell fate following DNA damage.

In my project, in addition to the detection of CLL tumours with bi-allelic *ATM* defects, I identified a proportion of tumours that had a mono-allelic *ATM* defect, either through a mutation or a deletion, in the presence of a second wild type *ATM* allele. Considering the poor clinical outcome of the whole group of tumours with an *ATM* mutation from the unselected cohort, I wanted to investigate whether there was any abnormality in the ATM function in those tumours that were found to have one mutant *ATM* allele and one wild type *ATM* allele. Similarly, from the cohort of tumours with an 11q deletion and hence loss on one *ATM* allele, I wanted to determine whether there was differential ATM activity between those tumours with an 11q deletion and a second mutant *ATM* allele from those with a second wild type *ATM* allele.

Having studied the effects of different combination of *ATM* defects on the cellular consequences to DNA damage with irradiation, I also wanted to investigate their effects on the response to chemotherapeutic drugs. Specifically, I wanted to study the role of ATM in the response to the purine analogue Fludarabine, which is currently a first-line therapy for CLL patients (Oscier D *et al*, 2004). Therefore, in this chapter I have investigated the role of fludarabine in the activation of the DNA damage response pathway and the effects of *ATM* mutations on the integrity of this pathway. I have also compared fludarabine-induced responses between tumours with two wild type *ATM* alleles, tumours with bi-allelic *ATM* defects and tumours with loss of one *ATM* allele through an 11q deletion with a remaining wild type *ATM* allele.

6.2 Functional assays to study the consequences of *ATM* mutations

6.2.1 ATM dependent phosphorylation responses

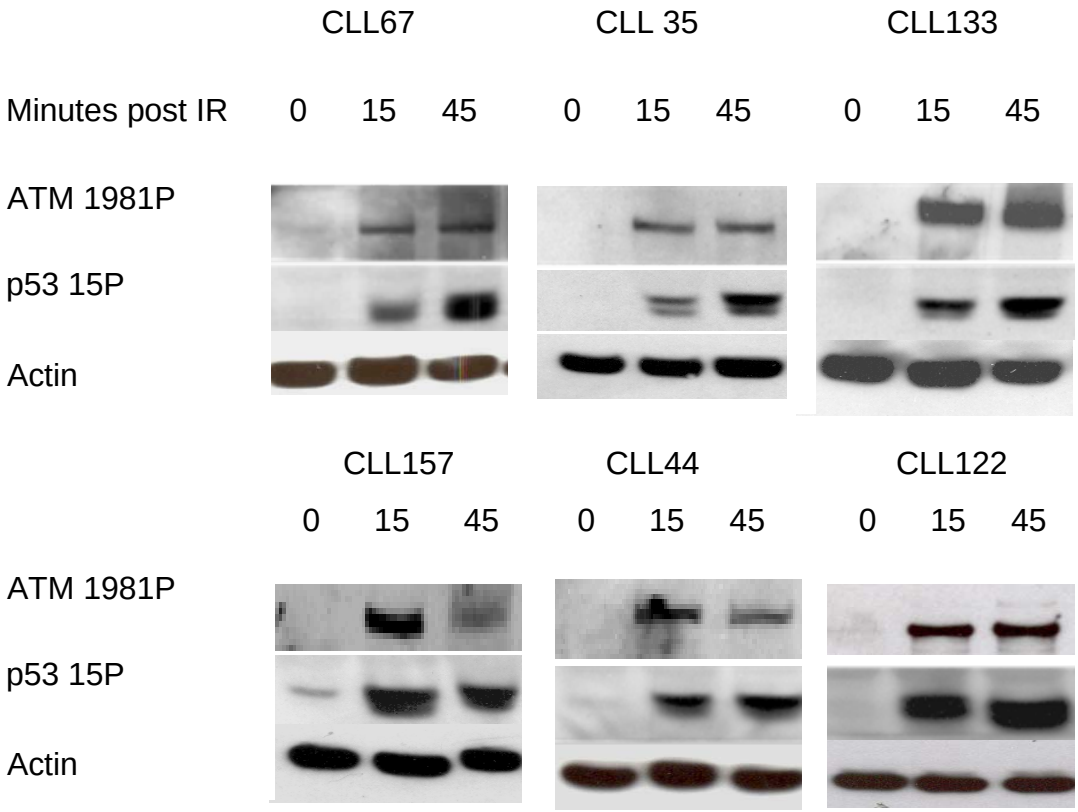
I investigated ATM dependent phosphorylation responses by measuring the change in the amount of an ATM target protein that is phosphorylated following DNA damage with ionising irradiation (IR). I studied several target substrates using commercial phospho-specific antibodies, including phosphorylated ATM on serine 1981, p53 on serine 15, SMC1 on serine 966 and Nbs 1 on serine 343. These substrates were chosen because they are known to be targets of ATM and also because they have been used in our laboratory both to evaluate the activity of various mutant ATM proteins as part of an in-vitro modelling system and to study residual ATM activity in certain AT patients (Kurz EU, Lees-Miller S, 2004 and unpublished data).

The activation of ATM is known to occur rapidly following the induction of DNA DSBs in response to IR (Bakkenist CJ, Kastan MB, 2003). Therefore, in the first instance, I assessed ATM dependent phosphorylation responses at 15 and 45 minutes after IR in a range of CLL tumours that were known to have two wild type *ATM* alleles. For these experiments, I used antibodies to phosphorylated ATM and p53, and compared the level of the respective phosphorylated proteins after IR with the level in the un-irradiated cells (time 0). I showed that following IR, the level of ATM phosphorylated on serine 1981 was markedly increased by 15 minutes and in the majority of tumours remained phosphorylated at 45 minutes. The level of p53 phosphorylated on serine 15 was also increased by 15 minutes after IR but increased further at 45 minutes (Figure 6.1). Altogether, I assessed these phosphorylation

Figure 6.1

There is phosphorylation of ATM on serine 1981 and p53 on serine 15 at 15 and 45 minutes following DNA damage with ionizing irradiation (5Gy) in CLLs 67, 35, 133, 157, 44 and 122, which all had two wild type *ATM* alleles. Actin is used as a loading control. This pattern of response was seen in all the tumours that were analysed that had two wild type *ATM* alleles.

Figure 6.1


CLL67
CLL 35
CLL133
Minutes post IR
0 15 45
0 15 45
0 15 45
ATM 1981P
p53 15P
Actin
CLL157
CLL44
CLL122
0 15 45
0 15 45
0 15 45
ATM 1981P
p53 15P
Actin

responses in 17 independent CLL tumours, which were all known to have two wild type *ATM* alleles and confirmed this pattern of response in all the tumours tested.

In 5 CLL tumours with two wild type *ATM* alleles I went on to investigate the phosphorylation of the additional ATM targets SMC1 and Nbs1 after IR. In all these tumours, phosphorylation of these downstream targets could also be demonstrated at 15 and 45 minutes after DNA damage with IR and the pattern was similar to that seen with phosphorylated p53 (Figure 6.2).

Having confirmed the presence of these phosphorylation responses in CLL tumours at these early time points after IR, I next investigated the responses in tumours with *ATM* mutations. In this project, I had identified 23 tumours that would be predicted to have loss of both functional *ATM* alleles, either through 2 *ATM* mutations (3 tumours) or through one *ATM* mutation and one allelic deletion (20 tumours). Viable cells were available in 12 of these cases and I was therefore able to study the phosphorylation responses in these tumours. In these experiments, I compared the DNA damage induced responses of tumours with *ATM* mutations with the responses of CLL tumours with two wild type *ATM* alleles and measured responses at 15 and 45 minutes after IR. I aimed to assess the phosphorylation of at least two substrates for each tumour.

I was able to demonstrate that there was impairment in the ATM dependent phosphorylation responses in all twelve tumours tested. As illustrated in Figure 6.3, there is impairment of phosphorylation of ATM and SMC1 or Nbs1 in the seven CLL tumours with an *ATM* mutation and an 11q deletion (CLLs 166, 198, 57, 180, 183, 152 and 182) compared to the CLL

Figure 6.2

There is also phosphorylation of additional ATM substrates following IR (5Gy), namely, SMC1 at serine 966 and Nbs1 at serine 343 in CLLs 53, 122, 42, and 61 and SMC1 at serine 966 in CLL 24. These tumours all have two wild type *ATM* alleles. The baseline level of the proteins SMC1, Nbs1 and ATM are shown in order to demonstrate the change in the proportion of cellular protein that is phosphorylated after DNA damage.

Figure 6.2

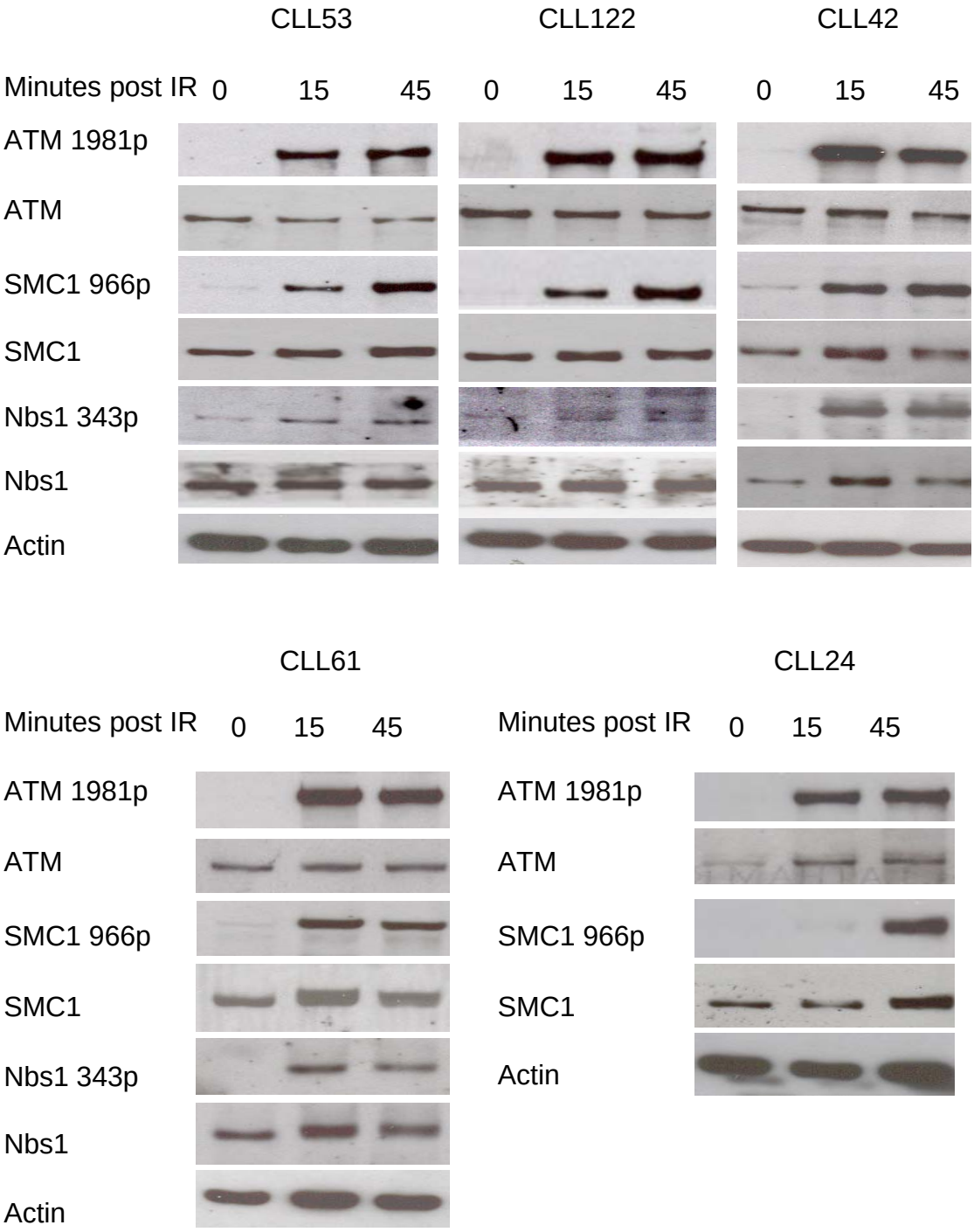

CLL53
CLL122
CLL42
Minutes post IR
0
15
45
ATM 1981p
ATM
SMC1 966p
SMC1
Nbs1 343p
Nbs1
Actin
CLL61
CLL24

Figure 6.3a and 6.3b

There is impairment in the phosphorylation of ATM on serine 1981 and SMC1 on serine 966 following IR (5Gy) in CLLs 166, 198, 57, 180, 183, 152 and 182, which all have loss of both *ATM* alleles through the combination of an 11q deletion and an *ATM* mutation, in comparison to the phosphorylation responses demonstrated in CLLs 122, 53, 42 and 24, which all have two wild type *ATM* alleles. A similar pattern of impairment of phosphorylation of Nbs1 is also seen in CLLs 166 and 198.

There is reduced total ATM protein expression in the CLL tumours with bi-allelic *ATM* defects (CLLs 166, 198, 57, 180, 183, 152, 182) but the levels of the proteins SMC1 (and Nbs1) are comparable between all the tumours regardless of the status of *ATM*. Therefore, the reduced level of phosphorylated ATM is due to both a decrease in the amount and function of the protein, but the reduced level of phosphorylated SMC1 (and Nbs1) is due to reduced function of ATM in the CLLs with bi-allelic *ATM* defects.

Figure 6.3a

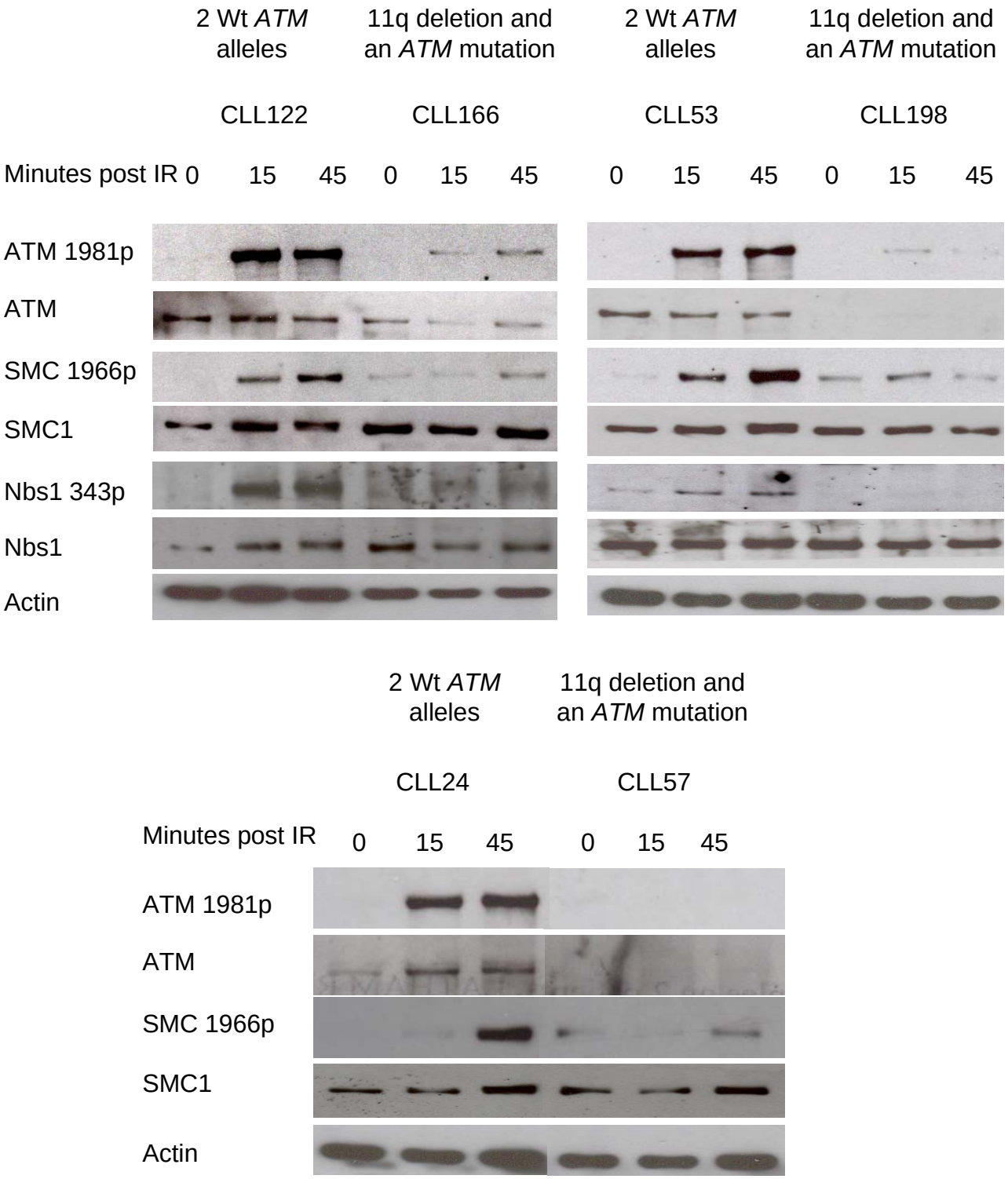
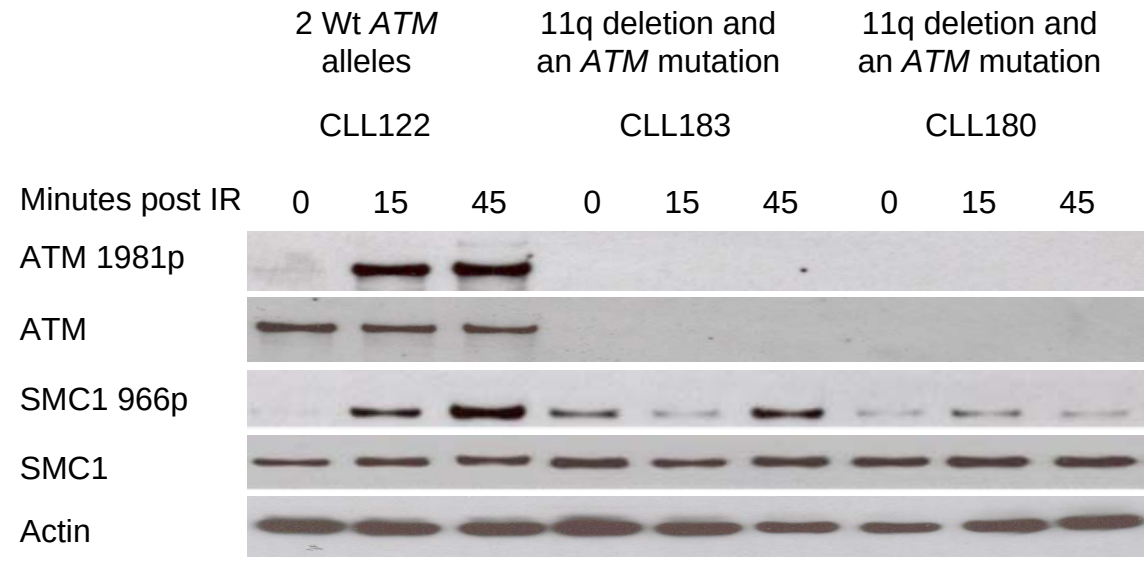
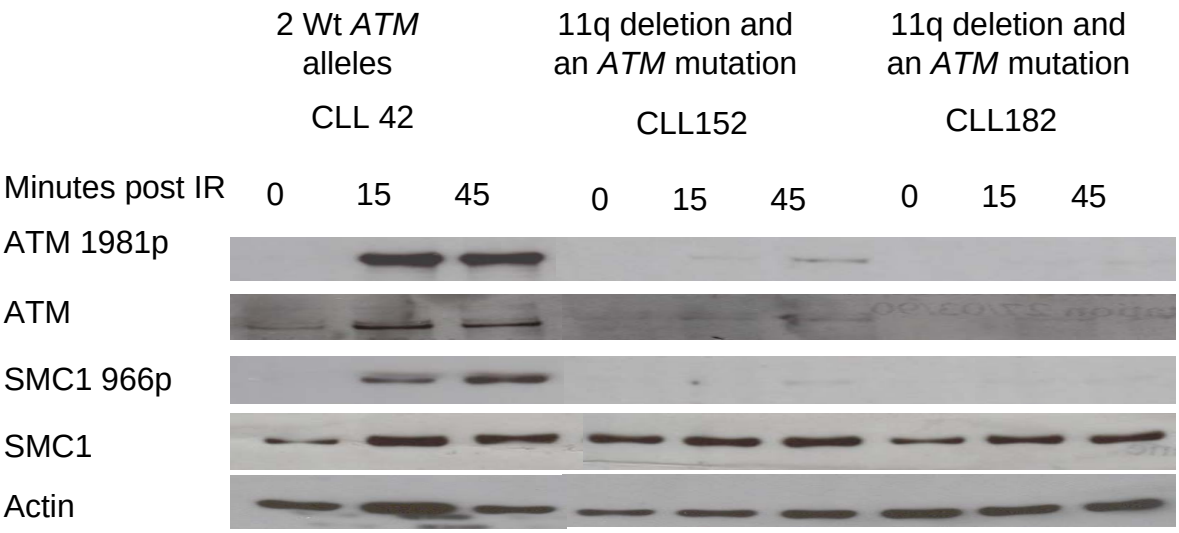

2 Wt *ATM* alleles
11q deletion and an *ATM* mutation
2 Wt *ATM* alleles
11q deletion and an *ATM* mutation
CLL122
CLL166
CLL53
CLL198
Minutes post IR 0 15 45 0 15 45 0 15 45 0 15 45
ATM 1981p
ATM
SMC 1966p
SMC1
Nbs1 343p
Nbs1
Actin
2 Wt *ATM* alleles
11q deletion and an *ATM* mutation
CLL24
CLL57
Minutes post IR 0 15 45 0 15 45
ATM 1981p
ATM
SMC 1966p
SMC1
Actin

Figure 6.3b


2 Wt *ATM* alleles
11q deletion and an *ATM* mutation
11q deletion and an *ATM* mutation
CLL122
CLL183
CLL180
Minutes post IR
0 15 45 0 15 45 0 15 45
ATM 1981p
ATM
SMC1 966p
SMC1
Actin


2 Wt *ATM* alleles
11q deletion and an *ATM* mutation
11q deletion and an *ATM* mutation
CLL 42
CLL152
CLL182
Minutes post IR
0 15 45 0 15 45 0 15 45
ATM 1981p
ATM
SMC1 966p
SMC1
Actin

tumours with two wild type *ATM* alleles at 45 minutes after DNA damage with irradiation. I also demonstrated an impairment of phosphorylation of Nbs1 in CLLs 166 and 198. The remaining 2 tumours (CLLs 07 and 15) with an *ATM* mutation and an 11q deletion that were investigated in this manner demonstrated a similar pattern of impairment of phosphorylation of downstream substrates (data not shown). There was also impairment of phosphorylation of ATM and p53 in the three tumours (CLLs 11, 96 and 77) that were predicted to have bi-allelic *ATM* defects as a consequence of two or more mutations (Figure 6.4). The choice of substrates that I assessed was partly influenced by the efficacy of the phosphorylated antibodies. In addition, in the initial experiments, I only used antibodies to phosphorylated ATM and p53 and in subsequent experiments I also used antibodies to phosphorylated SMC1 and Nbs1.

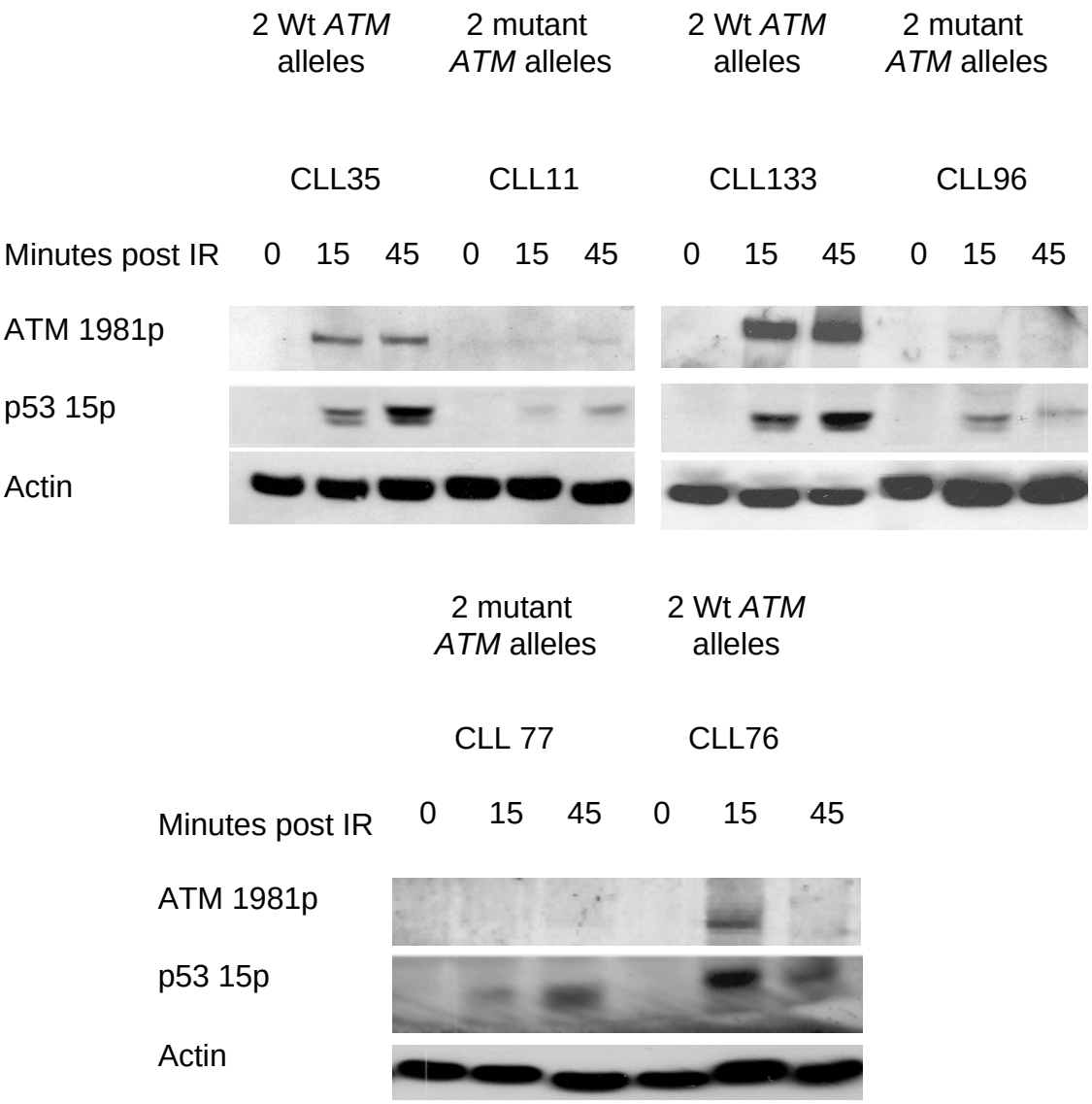
In all the tumours that I investigated which had bi-allelic *ATM* defects there was invariably very low level ATM activity as assessed by these DNA damage response phosphorylation assays. Nevertheless, the retention of some residual phosphorylation of ATM target substrates could be a consequence of either the ATM activity present in any non-tumour cells in the sample or in the presence of a subclone of tumour cells that did not carry the 11q deletion or the *ATM* mutation. Finally it is also possible that certain mis-sense *ATM* mutations could encode for a mutant ATM protein that had some residual retained ATM activity.

In this study, amongst the subgroup of 11q deleted tumours that had an additional *ATM* mutation, the chromosome 11q deletion was present in the majority of the tumour cell population in the majority of cases. Amongst the

Figure 6.4

There is reduced phosphorylation of ATM on serine 1981 and p53 on serine 15 following IR (5Gy) in CLLs 11, 96 and 77, which have two or more *ATM* mutations, in comparison to CLLs 35, 133 and 76, which have two wild type *ATM* alleles. Thus the mutations have resulted in the inactivation of the ATM protein in CLLs 11, 96 and 77 and this suggests that there were bi-allelic *ATM* defects in these tumours.

Figure 6.4


2 Wt *ATM* alleles
2 mutant *ATM* alleles
2 Wt *ATM* alleles
2 mutant *ATM* alleles
CLL35
CLL11
CLL133
CLL96
Minutes post IR
0 15 45 0 15 45 0 15 45 0 15 45
ATM 1981p
p53 15p
Actin
2 mutant *ATM* alleles
2 Wt *ATM* alleles
CLL 77
CLL76
Minutes post IR
0 15 45 0 15 45
ATM 1981p
p53 15p
Actin

nine cases in which I performed functional analysis, there was greater than 90% of tumour cells with an 11q deletion in 6 cases (CLLs 166, 180, 07, 15, 57 and 152), 84% and 69% of cells had an 11q deletion in CLLs 183 and 182 respectively, while the percentage of cells with an 11q deletion was not known in CLL198. However, as discussed later, there were also three 11q-deleted tumours (CLLs 192, 203 and 173) in which an *ATM* mutation had been detected where the chromosome 11q deletion was present in less than 50% of cells (Table 5.6). Viable cells for functional testing were not available in any of these three tumours but in these cases one might expect higher levels of ATM activity as a consequence of a significant number of cells that do not carry the 11q deletion. In all the twelve tumours with bi-allelic *ATM* defects where I performed functional analysis, there was very low level ATM activity. This therefore suggests that the *ATM* mutations in these tumours were also likely to be present in either the entire clone or a large subclone and that these mutations were indeed exerting a pathogenic effect.

In conclusion, therefore, on the basis of all twelve tumours tested in this study, I was able to show that the measurement of these ATM dependent phosphorylation responses can readily discriminate between tumours with two wild type *ATM* alleles and tumours with bi-allelic abnormalities of the *ATM* gene due to either two mutations or a mutation and a deletion. Therefore, this assay should allow the identification of tumours with abnormal ATM function as a consequence of *ATM* genetic defects.

6.2 Up regulation of p53 and expression of p21

The consequences of the ATM dependent phosphorylation of p53 on serine 15 and additional residues include the up regulation of the total p53 protein level in the nucleus. Here p53 can act as a transcription factor and after DNA damage there is a p53 dependent increase in the expression of the p21 protein. Therefore, an alternative way to investigate ATM activity in CLL tumour cells is through the measurement of the change in total levels of p53 and p21 after DNA damage, and, indeed, such an assay has been previously developed (Pettitt A *et al*, 2001). I assessed p53 and p21 levels following irradiation in 84 of the tumours that I had found to have wild type *ATM* and *TP53*. In 78 of these 84 tumours I observed low or undetectable levels of p53 and p21 at time 0, and up regulation of both proteins at 8 hours after IR in a pattern that is consistent to previous reports (Figure 6.5) (Pettitt A *et al*, 2001, Stankovic T *et al*, 2002).

Interestingly, in 6 tumours, there was reduced or absent induction of p21 expression, despite evidence of up regulation of p53. One explanation to account for these findings might be that the tumours had *ATM* mutations that had escaped detection by the DHPLC method. In 3 of these 6 CLL tumours, I was also able to examine the early ATM dependent phosphorylation responses after IR and in all three cases (CLLs 67, 153 and 39), there was evidence of phosphorylation of ATM on serine 1981 and of p53 on serine 15 in a similar manner to the other tumours tested that had two wild type *ATM* alleles (Figure 6.6). Therefore, this result indicated that there was no impairment in ATM activity in these 3 tumours. These results suggest that the ATM dependent phosphorylation events may correlate better with the genetic status of *ATM* than the measurement of downstream events. It is possible that

Figure 6.5

CLL tumours with wild type *ATM* and *TP53* genes showed up regulation of p53 and p21 levels at eight hours after DNA damage with IR (5Gy). This pattern of response is shown for 20 selected CLL tumours and was seen in a total of 78 out of 84 tumours that were analysed in this genetic category.

Figure 6.5

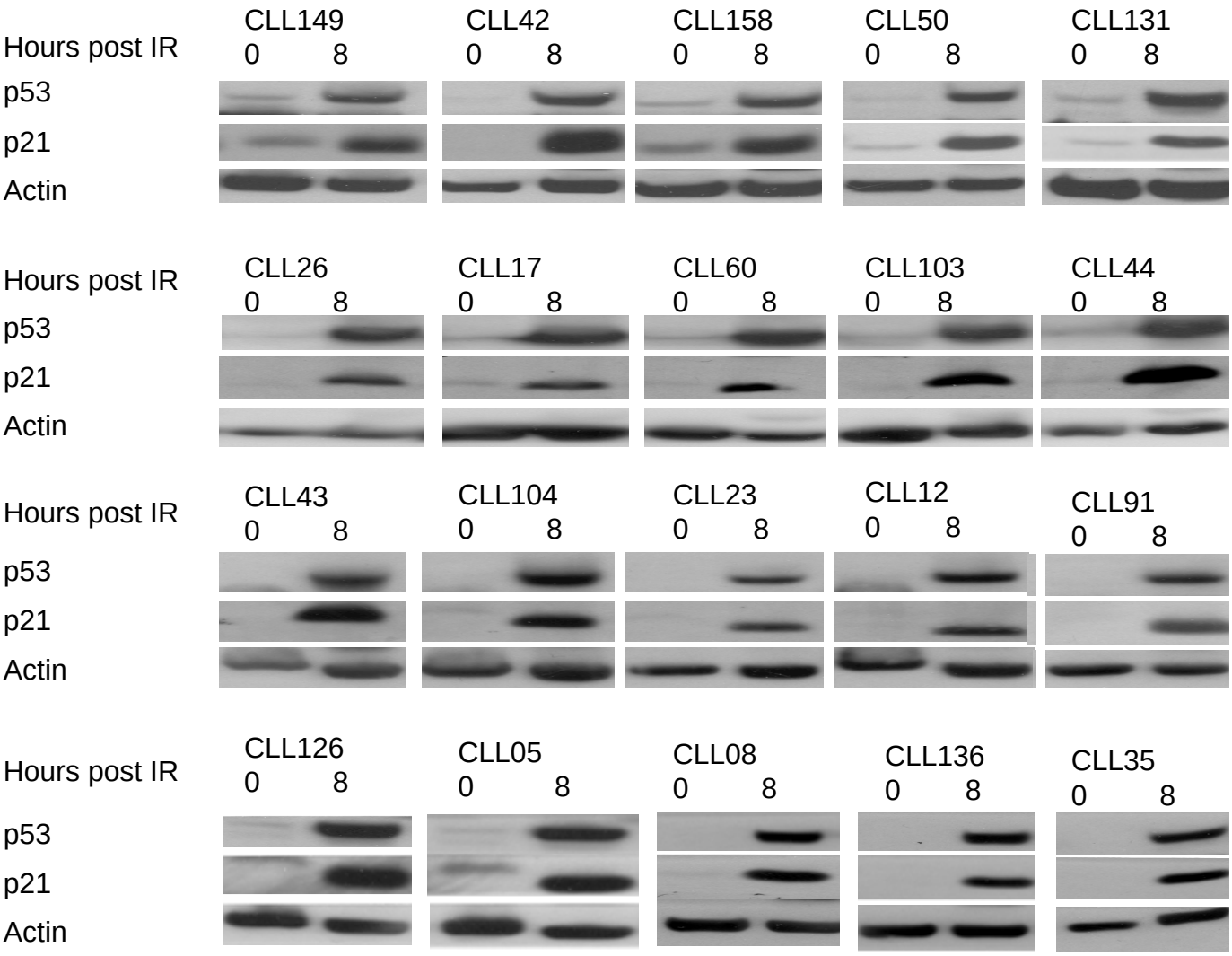
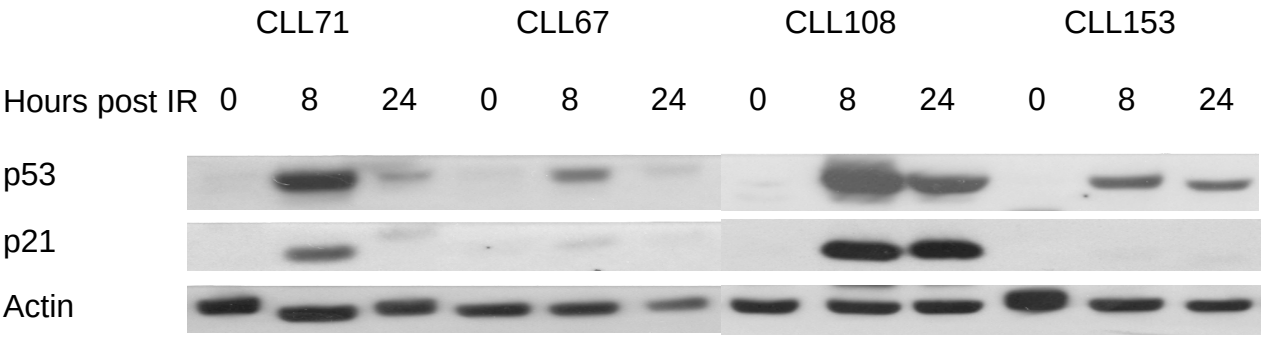

Hours post IR
CLL149
0 8
CLL42
0 8
CLL158
0 8
CLL50
0 8
CLL131
0 8
p53
p21
Actin
Hours post IR
CLL26
0 8
CLL17
0 8
CLL60
0 8
CLL103
0 8
CLL44
0 8
p53
p21
Actin
Hours post IR
CLL43
0 8
CLL104
0 8
CLL23
0 8
CLL12
0 8
CLL91
0 8
p53
p21
Actin
Hours post IR
CLL126
0 8
CLL05
0 8
CLL08
0 8
CLL136
0 8
CLL35
0 8
p53
p21
Actin

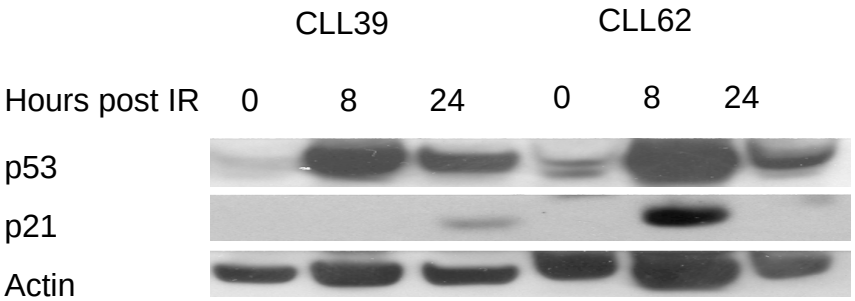
Figure 6.6

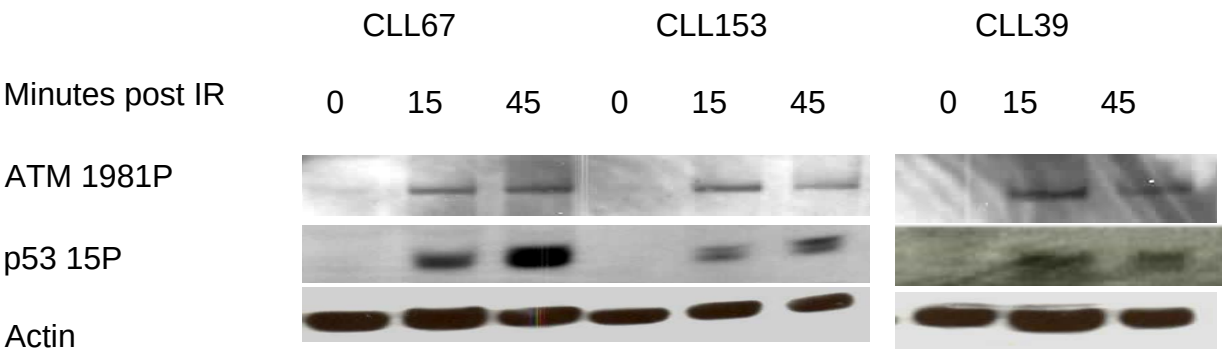
CLLs 67, 153 and 39 had wild type *ATM* and TP53 genes but showed an altered pattern of response. In these tumours there was reduced up regulation of p53 and absent induction of p21 at eight hours after IR (5Gy). This pattern was seen in 6 out of 84 tumours with wild type *ATM* and *TP53* genes.

Interestingly, CLLs 67, 153 and 39 all had retained phosphorylation responses after DNA damage. There was phosphorylation of ATM and p53 in all these tumours at 15 and 45 minutes after irradiation.

Figure 6.6


CLL71
CLL67
CLL108
CLL153
Hours post IR 0 8 24 0 8 24 0 8 24 0 8 24
p53
p21
Actin


CLL39
CLL62
Hours post IR 0 8 24 0 8 24
p53
p21
Actin


CLL67
CLL153
CLL39
Minutes post IR
0 15 45 0 15 45 0 15 45
ATM 1981P
p53 15P
Actin

these 6 tumours had a polymorphism in the p21 protein, and this has been previously shown to affect its expression after DNA damage (Carter A *et al*, 2006).

Having assessed the changes in p53 and p21 expression in tumours with two wild type *ATM* alleles, I subsequently assessed the responses in seven tumours with bi-allelic *ATM* defects. These included the three cases, CLLs 11, 96 and 77 (Figure 6.7) that had two or more *ATM* mutations and four cases, CLLs 57, 152, 07 and 15 that had one *ATM* mutation in conjunction with a deletion of chromosome 11q (Figure 6.13). In all seven cases there was reduced up regulation of p53 and p21 expression, in a pattern reminiscent to that described for tumours with *ATM* mutations in previous reports. For these tumours, there was good correlation between p53 and p21 responses and the ATM dependent phosphorylation responses. Therefore, the impairment of ATM activity in these tumours as measured by two assays validates the pathogenic nature of the mutations. In addition the effects of the *ATM* mutations on downstream cellular responses such as the up regulation of p53 and p21 will be predicted to impact on the induction of cell cycle arrest and the activation of apoptosis after DNA damage.

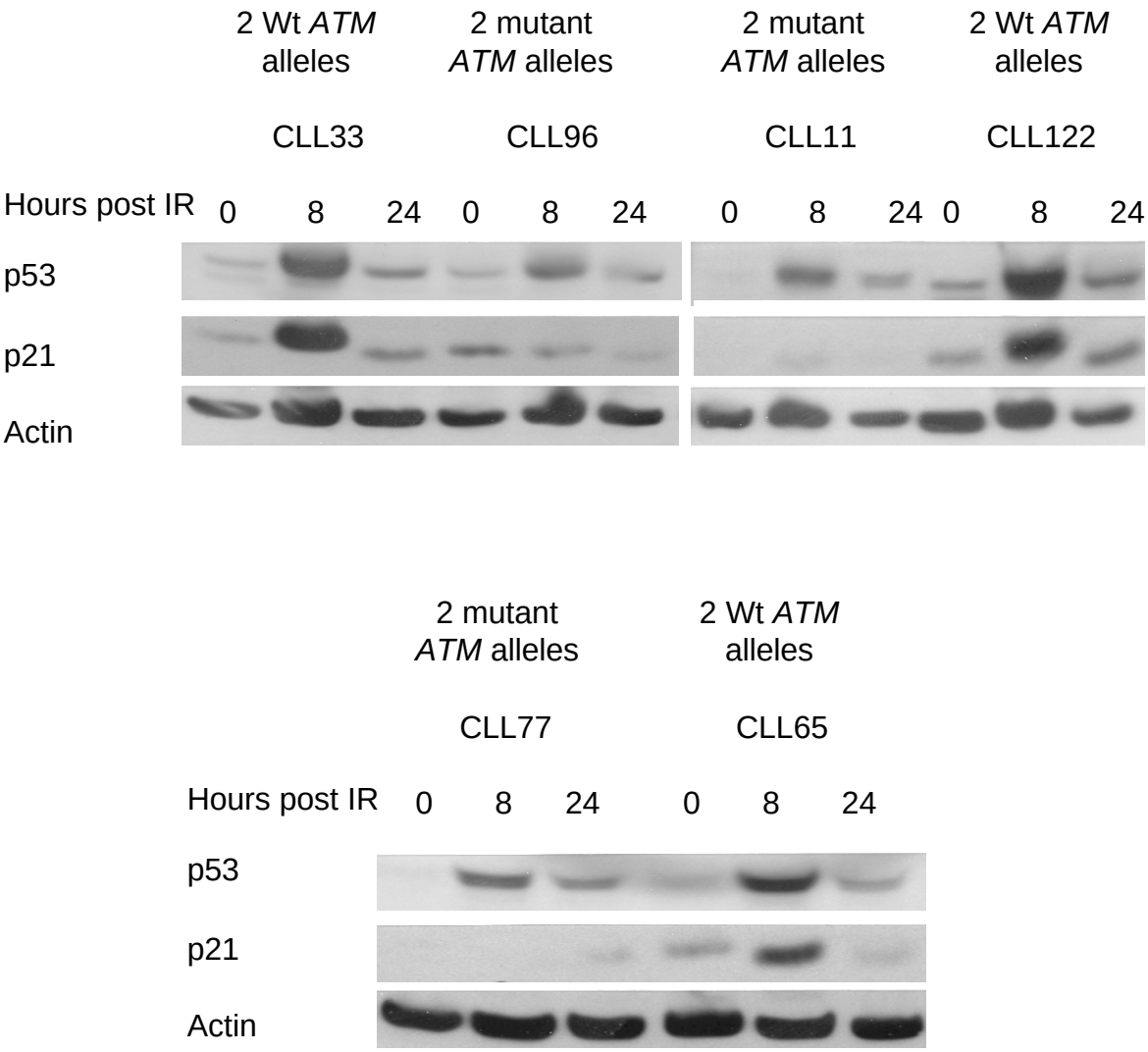
6.2.3 Induction of apoptosis

In order to assess the onset of apoptosis following DNA damage, I measured the cleavage of the DNA repair protein PARP1 following IR. PARP1 has been shown to be a target of the effector caspase 3 via the intrinsic apoptotic pathway (Norbury CJ, Hickson ID, 2001, Weston VJ et al; 2004). Initially, I wanted to confirm that PARP1 cleavage was indeed temporarily

Figure 6.7

CLLs 11, 96 and 77, which were predicted to have bi-allelic ATM defects as a consequence of mutations demonstrated impairment in the up regulation of p53 and the induction of p21 at 8 hours after IR (5Gy) compared to the proficient response patterns in CLLs 33, 122 and 65, which had wild type *ATM*.

Figure 6.7


2 Wt *ATM* alleles
2 mutant *ATM* alleles
2 mutant *ATM* alleles
2 Wt *ATM* alleles
CLL33
CLL96
CLL11
CLL122
Hours post IR
0 8 24 0 8 24 0 8 24 0 8 24
p53
p21
Actin
2 mutant *ATM* alleles
2 Wt *ATM* alleles
CLL77
CLL65
Hours post IR
0 8 24 0 8 24
p53
p21
Actin

synchronised with p53 mediated gene expression and activation of caspases, in CLL tumours. Therefore, I studied the changes in levels of p53 and p21 expression and also the levels of procaspase 7, cleaved caspase 3 and PARP1, in several tumours with wild type *ATM* at 8 and 24 hours after DNA damage with IR. I was able to demonstrate that the activation of p53-mediated apoptosis as indicated by the loss of procaspase 7 and the appearance of cleaved caspase 3, coincided with the loss of full length PARP1 and that these events followed the up regulation of p53 and the induction of p21 expression. This indicated that the cleavage of PARP1 was occurring via p53-mediated apoptosis in the CLL tumours (CLLs 53, 55 and 32) (Figure 6.8).

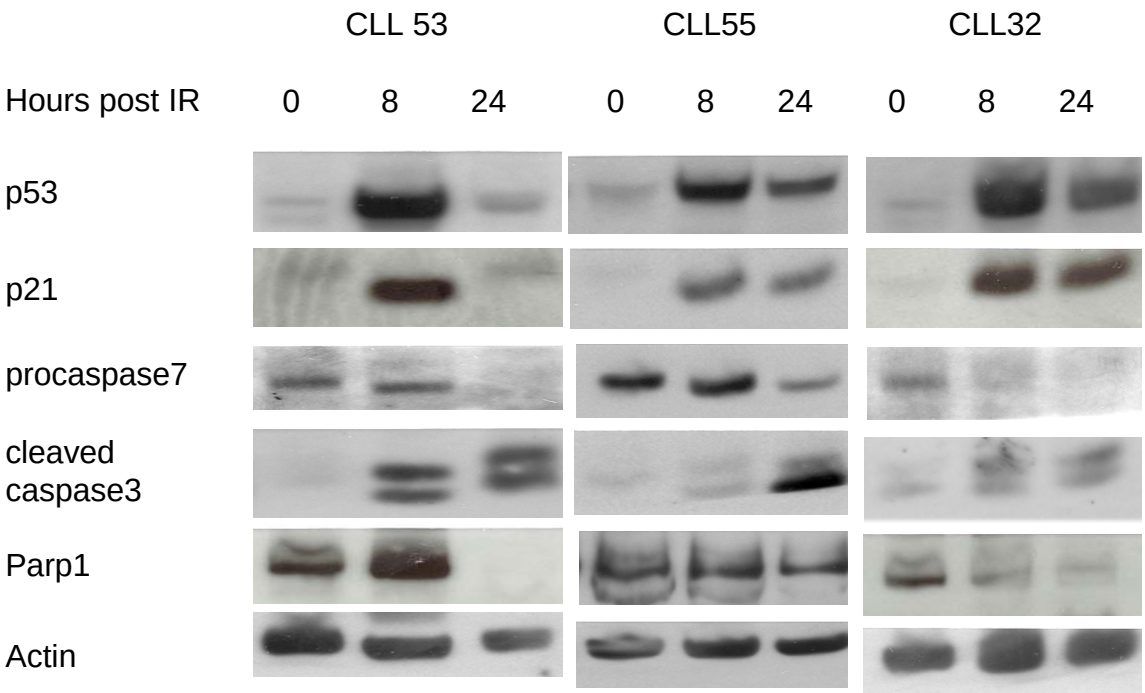
To evaluate the effects of *ATM* mutations on apoptosis, I compared the degree of PARP1 cleavage at 24 hours following IR in 12 CLLs with *ATM* mutations (and no mutation in the *TP53* gene) with 70 CLL tumours that were known to have both wild type *ATM* and *TP53* genes. Amongst these twelve tumours with *ATM* mutations, seven had bi-allelic *ATM* defects but ten had been demonstrated to have impairment of ATM function as indicated by the ATM-dependent phosphorylation responses following IR (see later). Loss of p53 function is known to lead to an apoptotic resistant phenotype in CLL tumours and, therefore, I also compared tumours with *ATM* mutations with the six tumours in which I had detected *TP53* mutations.

To obtain a quantitative and comparative measure of apoptosis between the CLL tumours, I calculated the percentage of the level of PARP1 at time 0, which remained at 24 hours following irradiation. Levels were corrected for actin to account for any differences in protein loading. An unchanged level of uncleaved PARP1 (100%) at 24 hours indicated an absence of apoptosis in a

Figure 6.8

The induction of the ATM / p53 response pathway following DNA damage with irradiation leads to the activation of apoptosis by the intrinsic pathway in CLL tumours. As illustrated for CLL53, 55 and 32 there is up regulation of p53 and p21 at 8 hours after irradiation. By 24 hours after IR (5Gy) there is evidence for the loss of procaspase 7 and the appearance of the active cleaved caspase 3. In addition there is also loss of the full length PARP1 by 24 hours after DNA damage.

Figure 6.8


CLL 53
CLL55
CLL32
Hours post IR
0
8
24
0
8
24
0
8
24
p53
p21
procaspase7
cleaved caspase3
Parp1
Actin

tumour and a low level of uncleaved PARP1 at 24 hours indicated that a high number of tumour cells had activated apoptosis.

Total absence of apoptosis was observed in 67% (4/6) of tumours with *TP53* mutations, 42% (5/12) of tumours with *ATM* mutations and also 16% (11/70) of *ATM*/*TP53* wild type tumours. The median values for residual PARP1 levels at 24 hours following irradiation in the genetic subgroups were 100% for *TP53* mutant tumours, 69% for *ATM* mutant tumours and 36% for *ATM/TP53* wild type tumours. These differences were statistically significant (Kruskal-Wallis test, p=0.019) (Figure 6.9 and 6.10).

Despite the significant differences between the genetic subgroups, there was a considerable degree of variation in the induction of apoptosis within each subgroup. Interestingly, twelve tumours that were *ATM/TP53* wild type showed complete resistance to PARP1 cleavage. These tumours had been shown to have normal up regulation of total p53 protein levels and the induction of p21 after DNA damage with IR. Therefore, this suggests that there might be a defect or alteration in targets that are downstream from the activation of p53, such as the Bcl-2 family of proteins. Indeed, in CLL tumours both Bcl2/Bax ratios and Mcl1 levels have been previously implicated in apoptosis resistance (Pepper C *et al*, 1996). Furthermore, there is also recent data suggesting that the up regulation of DNA repair activity may also lead to a DNA damage apoptotic resistance phenotype in CLL cells. This could also be independent of both the *ATM* and *TP53* mutation status (Derriano L *et al*, 2005).

Amongst the tumours with *ATM* mutations, there was also variation in the induction of apoptosis following IR compared to the upstream events, such as

Figure 6.9

Loss of full length PARP1 was assessed for tumours with *ATM* mutations, *TP53* mutations and *ATM /TP53* wild type genes as an indicator for the induction of apoptosis. CLLs 155, 80, 108, 34 and 33 all show loss of the full length PARP1 at 24 hours after DNA damage with IR (5Gy) compared to the level at time 0. Therefore these tumours have all activated apoptosis. By comparison there is impairment of the induction of apoptosis as indicated by the retention of full length PARP1 at 24 hours post IR in CLLs 130, 124 and 96. CLL 96 has three *ATM* mutations, CLL 124 has a *TP53* mutation and also an *ATM* mutation and CLL 130 has wild type *ATM* and *TP53* genes.

The percentage of the level of PARP1 at 0 that was present at 24 hours after irradiation, as measured by optical density, was calculated for each tumour. This value was then adjusted for any changes in Actin, which was used as a loading control. The values for optical density are shown beneath the protein band.

For example, in CLL80, the adjusted percentage of PARP1 at 24 hours was calculated:

Percentage of PARP1 at 24 hours = 2.61/35.47 x 100 = 7.4%

Percentage of Actin at 24 hours = 21.57/24.22 x 100 = 89%

Adjusted Percentage of PARP1 at 24 hours = 7.4/89 x100 = **8.3%**

Figure 6.9

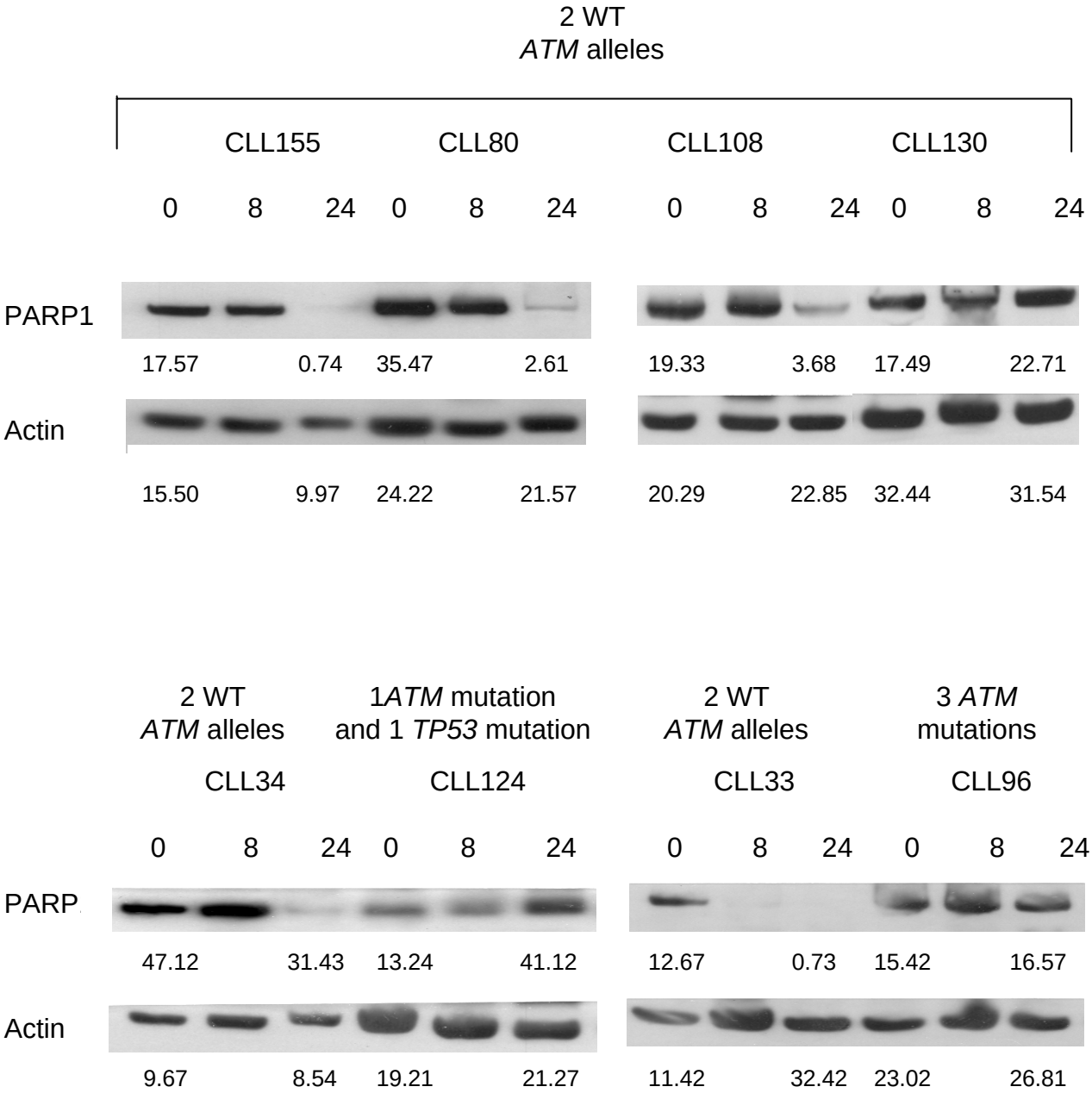
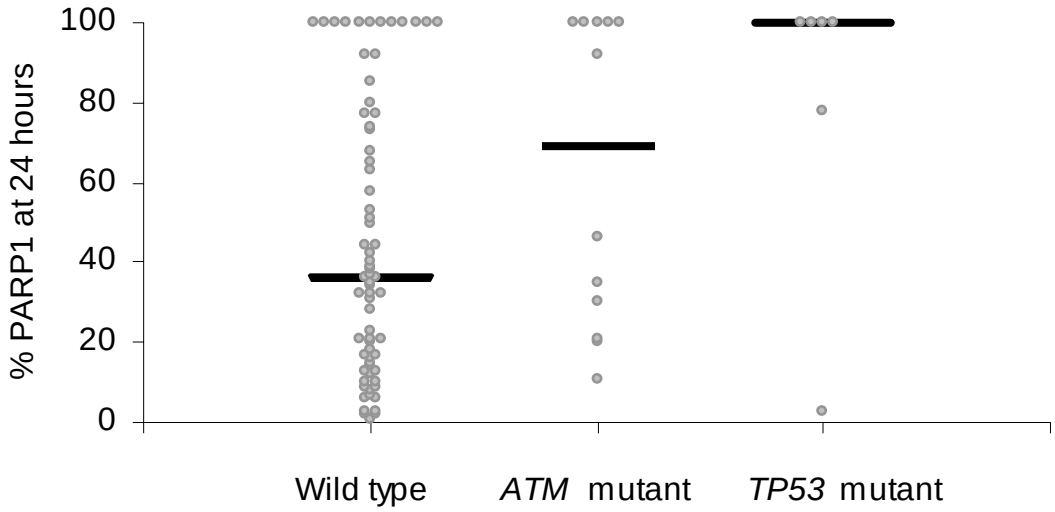

2 WT
ATM alleles
CLL155
CLL80
CLL108
CLL130
0 8 24 0 8 24 0 8 24 0 8 24
PARP1
17.57 0.74 35.47 2.61 19.33 3.68 17.49 22.71
Actin
15.50 9.97 24.22 21.57 20.29 22.85 32.44 31.54
2 WT
ATM alleles
1*ATM* mutation
and 1 *TP53* mutation
2 WT
ATM alleles
3 *ATM*
mutations
CLL34
CLL124
CLL33
CLL96
0 8 24 0 8 24 0 8 24 0 8 24
PARP
47.12 31.43 13.24 41.12 12.67 0.73 15.42 16.57
Actin
9.67 8.54 19.21 21.27 11.42 32.42 23.02 26.81

Figure 6.10

The adjusted percentage of full length PARP1 at 24 hours was compared between the three genetic subgroups, namely *ATM* mutant, *TP53* mutant and *ATM/TP53* wild type. The two tumours with an *ATM* mutation and a *TP53* mutation were included in the category of *TP53* mutant tumours. The median value for each genetic subgroup is shown.

Tumours with *TP53* mutations were the most resistant to apoptosis after DNA damage, and tumours with *ATM* mutations showed a pattern of resistance that was intermediate between those tumours with *TP53* mutations and those with wild type *ATM* and *TP53* genes. These differences were statistically significant (p=0.019). Notably there was a degree of variation in each genetic category and there were a number of tumours with wild type *ATM* and *TP53* genes that showed resistance to DNA damage induced apoptosis.

Figure 6.10


% PARP1 at 24 hours
100
80
60
40
20
0
Wild type
ATM mutant
TP53 mutant

ATM dependent phosphorylation of substrates and changes in the level of p53 and p21. Nevertheless, as a group, the tumours with *ATM* mutations did demonstrate an apoptotic resistant phenotype as measured by this PARP1 cleavage assay, which is in line with previous findings (Stankovic T *et al*, 2002).

In selected tumours with *ATM* wild type and *ATM* mutant genes, apoptosis was also assessed using an Annexin/Propidium iodide Fluorescent Activated Cell Sorting (FACS) method. This allowed the verification that the tumours with *ATM* mutations, identified in this cohort, did indeed have impaired activation of apoptosis. Fifteen *ATM* wild type tumours and seven *ATM* mutant tumours were analysed by this method and consistent with reduced PARP1 cleavage, *ATM* mutant tumours showed less efficient apoptosis. At 24, 48 and 72 hours following IR the mean levels of apoptosis among the *ATM* wild type tumours (after subtraction of any spontaneous apoptosis at these time points) were 36%, 53% and 73% respectively, compared to 22%, 31% and 39% in the *ATM* mutant tumours ($p = 0.017$, 0.015 and 0.04) (This FACS analysis was performed by Dr. A. Alvi).

These results from both assays confirm that CLL tumours with *ATM* mutations have an apoptotic resistant phenotype following DNA damage compared to *ATM* wild type CLL tumours but, nevertheless, indicate that the measurement of apoptosis following IR would be both a relatively non-specific and insensitive method to detect CLL tumours that harbour *ATM* mutations. Since the activation of apoptosis is the final downstream event in the DNA damage pathway it is perhaps not surprising that it will be influenced by

numerous cellular factors in addition to the activity of the upstream ATM protein.

6.3 Responses to IR in tumours with one remaining wild-type *ATM* allele

In this study, there were 44 tumours that were found to have an abnormality in a single *ATM* allele. In 33 cases this was due to a deletion of one copy of *ATM* through an 11q deletion but with a remaining wild type *ATM* allele, and, in 11 cases this was due to the presence of one mutant *ATM* allele and one wild type *ATM* allele. If ATM acts as a classical tumour suppressor gene, then it might be expected that the retention of one functional *ATM* allele would be adequate for normal ATM activity in the cell. I evaluated ATM activity in both these CLL tumour subgroups by assessing the responses to DNA damage with IR.

6.3.1 Tumours with a deletion of one *ATM* allele

I studied this category of tumours with a deletion of one *ATM* allele but a second wild type allele in order to determine whether heterozygosity at the ATM locus had any functional consequences in CLL tumours. Viable cells were available on 11 tumours and therefore in these tumours I measured the early ATM dependent phosphorylation responses following IR. Interestingly, this subgroup could be shown to have preserved ATM activity and the tumours demonstrated phosphorylation of downstream targets following IR, in a similar pattern to that seen for tumours with two wild type *ATM* alleles. Reduced levels of total ATM protein were apparent in most cases consistent with heterozygous ATM expression, which led to reduced levels of

phosphorylated ATM after IR. However, the proportion of the total ATM that was phosphorylated after IR was similar to that in the tumours with 2 wild type *ATM* alleles, indicating that the reduced level of phosphorylated ATM reflected the reduced ATM protein expression rather than impaired ATM function (Figures 6.11a and b).

Next, I wanted to assess whether the whole group of tumours with an 11q deletion could be divided into functional subgroups according to the status of the remaining *ATM* allele. Therefore, I quantified the phosphorylation responses, and compared the relative ATM activity of this subgroup of 11 tumours with an isolated 11q deletion, with the subgroup of 9 tumours with an 11q deletion and an *ATM* mutation, described previously. In order to quantify the response, I measured the change in the phosphorylated proportion of either p53 or SMC1 between time 0 and 45 minutes post IR for each tumour. I then expressed this value, for all the tumours with an 11q deletion, relative to the value for the control tumour on each western blot that had two wild type *ATM* alleles (Figure 6.12).

Compared to the very low ATM activity of all the tumours with two abnormal *ATM* alleles in this study, the tumours with an isolated 11q deletion all had higher levels of ATM activity. This was greater than 50% of the control tumour in all cases, and in some cases was equivalent or higher than that seen in the control tumours with two wild type *ATM* alleles. There was some variation in ATM activity amongst the tumours with the isolated 11q deletion and, since this assay was measuring relative activity, this may partly reflect a degree of variation between control samples.

Figure 6.11a and b.

CLL198 with an 11q deletion and an *ATM* mutation demonstrates a deficient phosphorylation response following DNA damage with IR (5Gy) in comparison to CLLs185 and 175 that show phosphorylation of SMC1 that is equivalent to that seen in CLL53 that has two wild type *ATM* alleles. Similarly, CLLs 37 and 25, with an 11q deletion and a second wild type *ATM* allele, demonstrate proficient phosphorylation of p53 after IR that is equivalent to the response in CLL13, with two wild type *ATM* alleles. Thus the retention of one wild type *ATM* allele is sufficient for normal ATM activity in these tumours.

The change in the proportion of SMC1 that is phosphorylated, as assessed by optical density, was calculated for each tumour with an 11q deletion and expressed relative to the change in this proportion in the tumour with two wild type *ATM* alleles on the same blot.

For CLL185:

Change in percentage of SMC1 which is phosphorylated between 45 and 0 minutes = ((29.12/34.43) x 100) – ((0.02/15.32) x 100)) = 84%

For CLL 53:

Change in percentage of SMC1 which is phosphorylated between 45 and 0 minutes = ((16.21/22.91) x 100) – ((0.23/13.94) x 100)) = 69.5%

Relative change in percentage of phosphorylated SMC1 for CLL185:

= 84/69.5 x 100 = **121%**

Figure 6.11a

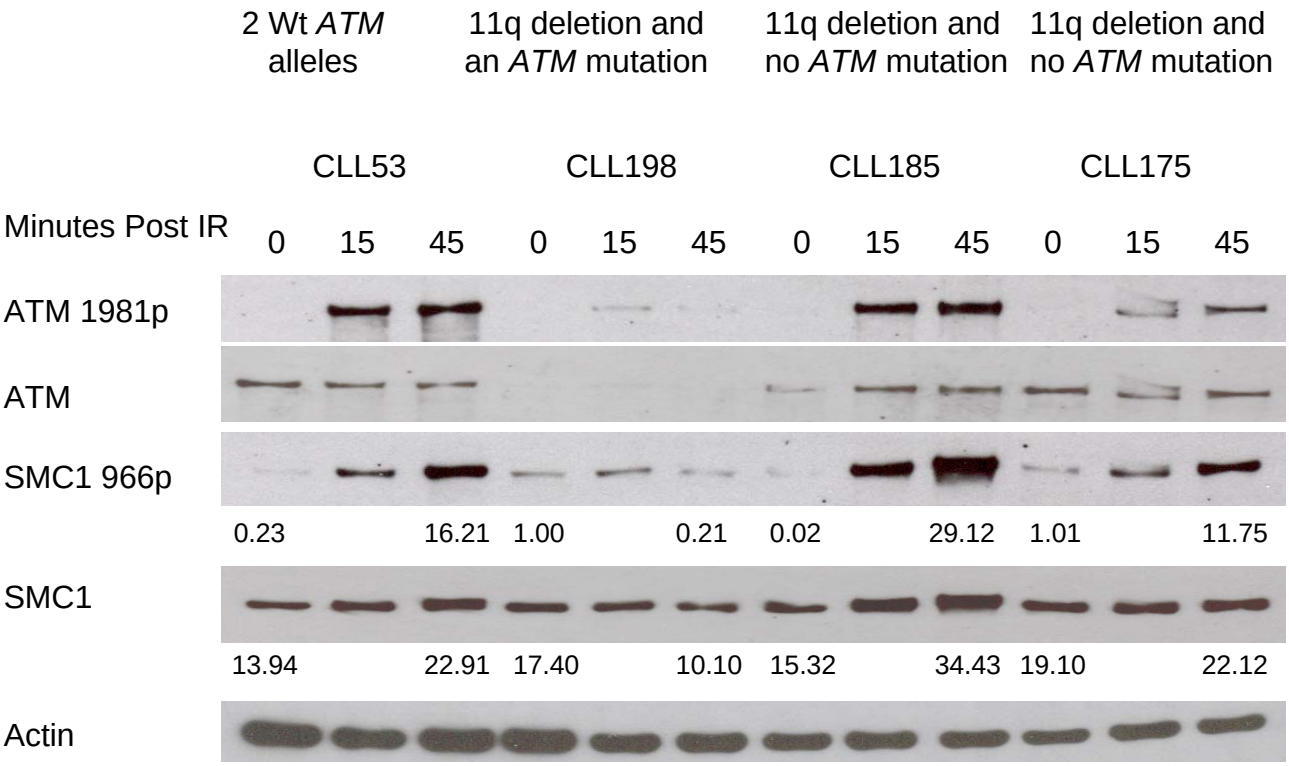

2 Wt *ATM* alleles
11q deletion and an *ATM* mutation
11q deletion and no *ATM* mutation
11q deletion and no *ATM* mutation
CLL53
CLL198
CLL185
CLL175
Minutes Post IR
0 15 45 0 15 45 0 15 45 0 15 45
ATM 1981p
ATM
SMC1 966p
0.23 16.21 1.00 0.21 0.02 29.12 1.01 11.75
SMC1
13.94 22.91 17.40 10.10 15.32 34.43 19.10 22.12
Actin

Figure 6.11b

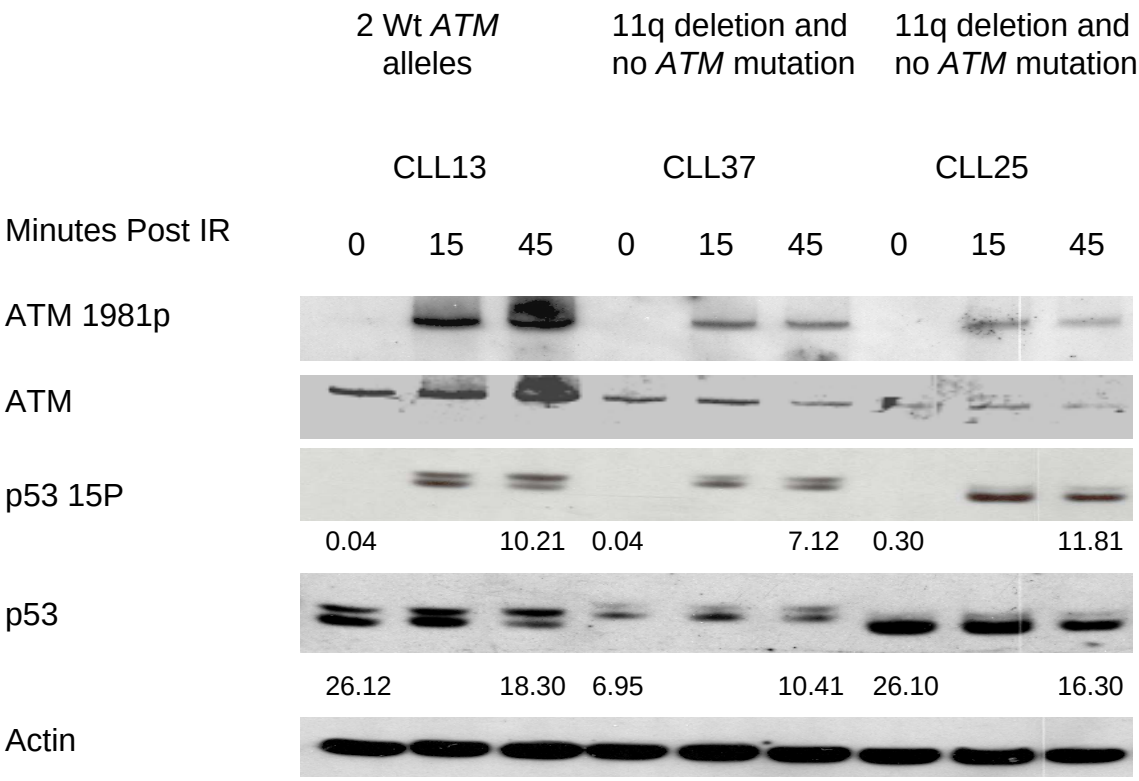
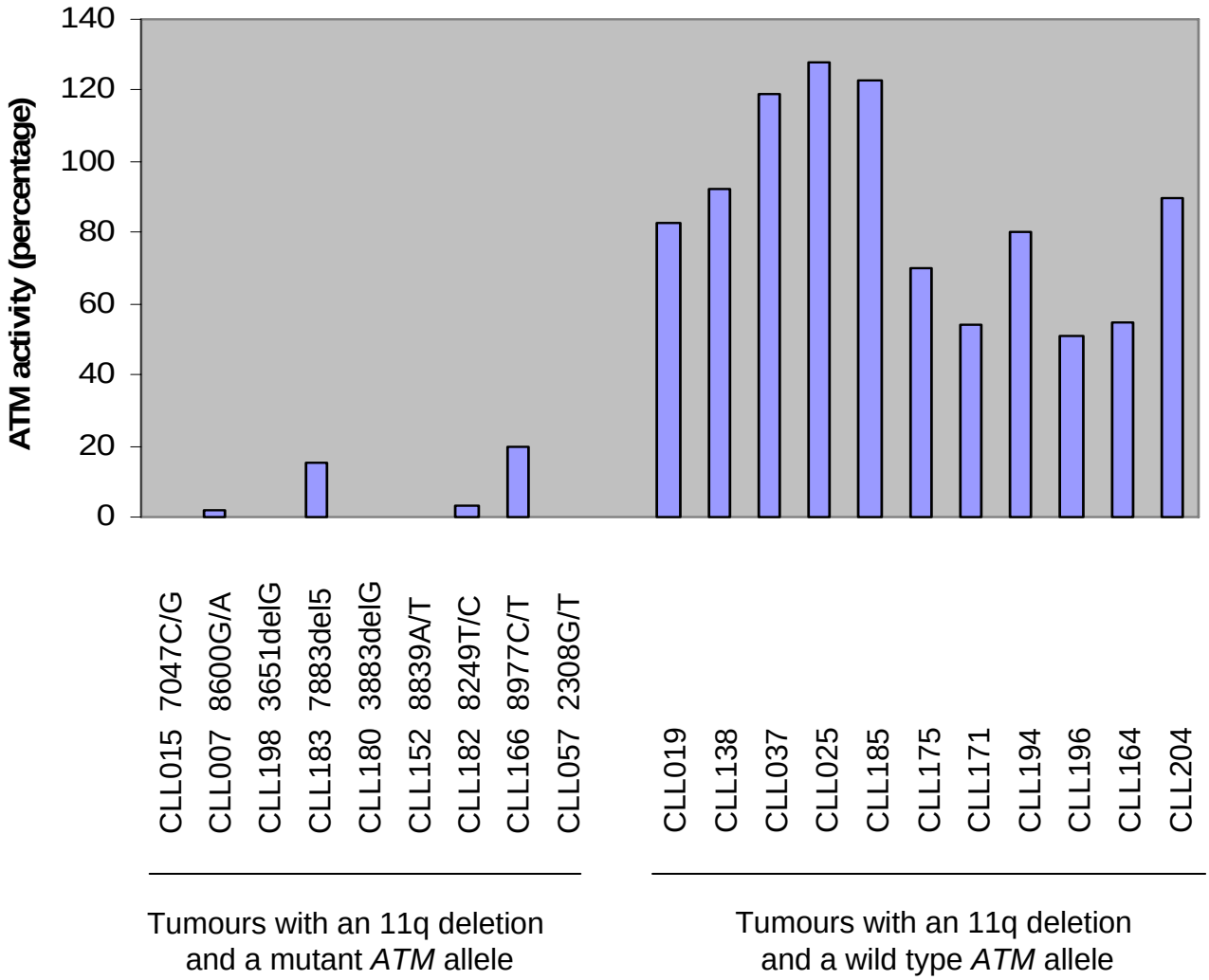

2 Wt *ATM* alleles
11q deletion and no *ATM* mutation
11q deletion and no *ATM* mutation
CLL13
CLL37
CLL25
Minutes Post IR
0 15 45 0 15 45 0 15 45
ATM 1981p
ATM
p53 15P
0.04 10.21 0.04 7.12 0.30 11.81
p53
26.12 18.30 6.95 10.41 26.10 16.30
Actin

Figure 6.12

The relative ATM activity in the tumours with an 11q deletion is demonstrated. Tumours with an 11q deletion and an *ATM* mutation all had low levels of ATM activity. By comparison, tumours with an 11q deletion and a second wild type *ATM* allele had retained ATM activity.

Figure 6.12


ATM activity (percentage)
140
120
100
80
60
40
20
0
CLL015 7047C/G
CLL007 8600G/A
CLL198 3651delG
CLL183 7883del5
CLL180 3883delG
CLL152 8839A/T
CLL182 8249T/C
CLL166 8977C/T
CLL057 2308G/T
CLL019
CLL138
CLL037
CLL025
CLL185
CLL175
CLL171
CLL194
CLL196
CLL164
CLL204
Tumours with an 11q deletion and a mutant *ATM* allele
Tumours with an 11q deletion and a wild type *ATM* allele

These results indicated that the retention of one wild type *ATM* allele is sufficient for normal ATM activity in CLL tumour cells. However, with this type of semi-quantitative assay, it was not possible to exclude with certainty that heterozygosity at the ATM locus might not exert some effect on ATM activity. Nevertheless, it was possible to conclude that all these tumours with an isolated 11q deletion had much higher level of ATM activity than the group with a second mutant *ATM* allele and that the two subgroups could be readily distinguished.

Interestingly, there was a much greater variation in the percentage of cells in the CLL clone that carried the 11q deletion amongst this subgroup of tumours that did not have an *ATM* mutation in the second allele compared to the subgroup in which there was also an *ATM* mutation (Tables 5.6 and 5.7). Taking the two groups as a whole, the mean and median values for the proportion of the CLL clone that carried an 11q deletion was 87 and 78.25%, respectively, for the subgroup with an additional *ATM* mutation, compared to 50 and 57.2%, respectively, for the subgroup with a second wild type *ATM* allele. Notably, amongst the tumours in the subgroup of 11q-deleted tumours with wild type *ATM*, I was able to demonstrate high levels of ATM activity in several tumours that had greater than 75% of CLL cells carrying the 11q deletion [CLL185 (90%), CLL19 (95%), CLL25 (97%), CLL138 (80%), CLL 37 (75%)]. This indicated that it was indeed the residual wild type *ATM* allele that was accounting for the ATM activity in these assays rather than the presence of a large subclone of cells with two wild type *ATM* alleles.

By comparison, three of the tumours that I tested for ATM activity from this subgroup had only a small clone of cells with the 11q deletion [CLL171 (10%),

CLL175 (11%), CLL194 (23%)]. All these tumours also had retained ATM activity but, given the small size of the sub-clone with the 11q deletion, this pattern might be independent of the mutation status of the second *ATM* allele. Therefore, this type of assay might be unable to distinguish differences in ATM activity between 11q-deleted tumours, with and without *ATM* mutations, in those cases where there is only a small subclone that carries the chromosome deletion. However; as indicated in this study, those 11q-deleted tumours that also have *ATM* mutations invariably have a high percentage of cells carrying the 11q deletion (median 87%) and therefore these would be predicted to have impairment of ATM activity, which would thus be identifiable with this type of assay.

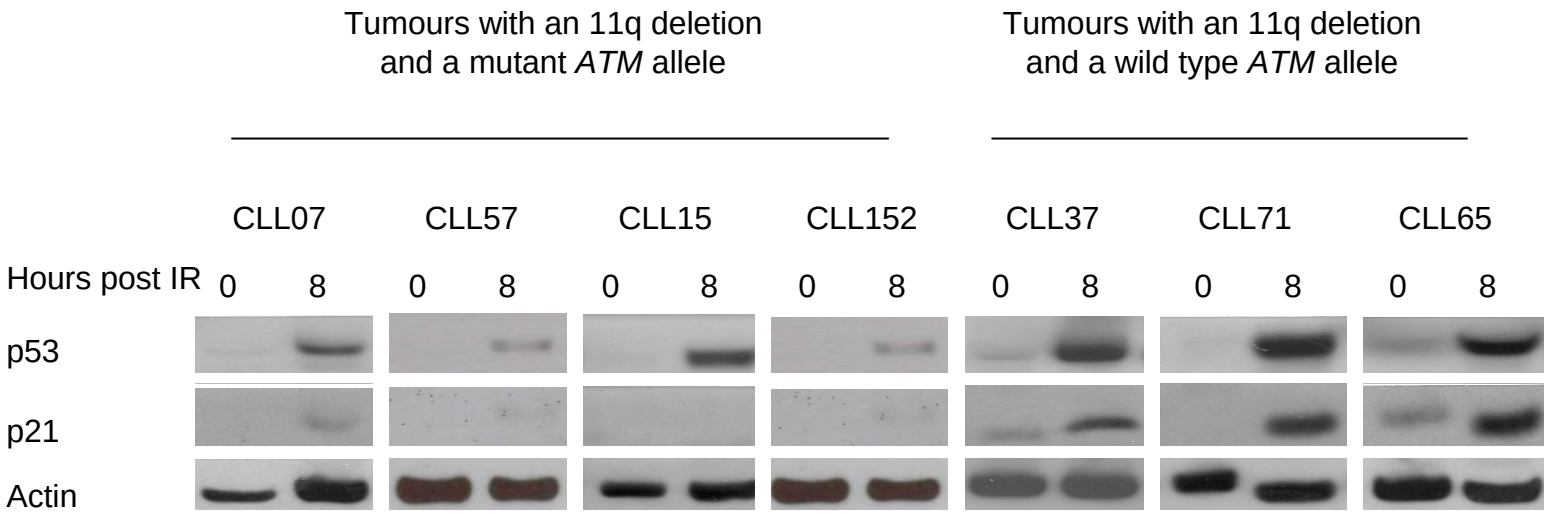
I also tested the up regulation of p53 and induction of p21 expression in three tumours with a deletion of 11q and a second wild type *ATM* allele [CLL65 (91% 11q deletion), CLL71 (60%) and CLL37 (75%)] and compared these responses to four tumours with a deletion of 11q and a second mutant *ATM* allele [CLL07 (92%), CLL15 (95%), CLL57 (90%), CLL152 (99%)]. Once again, the CLL tumours with loss of an *ATM* allele but with a second wild type *ATM* allele showed a pattern of up regulation of p53 and induction of p21 expression at 8 hours post IR that was equivalent to that seen in tumours with two *ATM* wild type alleles. By comparison the four tumours with a second mutant *ATM* allele had a deficient response pattern (Figure 6.13).

Taken together, the results in this study indicate that CLL tumours with a deletion of chromosome 11q can be divided into two functional groups that are dependent upon the mutation status of the remaining *ATM* allele.

Figure 6.13

There was also differential up regulation of p53 and induction of p21 expression following DNA damage with IR (5Gy) in CLL tumours with an 11q deletion depending on the status of the second *ATM* allele. CLLs 07, 57, 15 and 152 with an 11q deletion and an *ATM* mutation had impairment in the up regulation of p53 and induction of p21. In comparison, CLLs 37, 71 and 65 with an 11q deletion and a wild type second *ATM* allele all demonstrated a proficient response.

Figure 6.13


Tumours with an 11q deletion and a mutant *ATM* allele
Tumours with an 11q deletion and a wild type *ATM* allele
CLL07
CLL57
CLL15
CLL152
CLL37
CLL71
CLL65
Hours post IR
0
8
p53
p21
Actin

6.3.2 Tumours with a mutation in one *ATM* allele

There were eleven tumours with a single *ATM* mutation that had not been previously found to have a deletion of chromosome 11q in this study. These tumours are therefore also heterozygous at the ATM locus but as a result of a mutation rather than an allelic deletion. Viable cells were available in 9 of these cases and once again I assessed tumour cell responses to IR.

Interestingly, I found that there was a variable pattern of ATM activity in this group of tumours. In four cases (CLLs 119, 75, 69, 93), there was a marked reduction in ATM activity reminiscent of the situation in the tumours with loss of functional both *ATM* alleles (Figure 6.14). The mutations that gave rise to this pattern of impaired phosphorylation of ATM targets were, CLL119 (9022C/T), CLL75 (1009C/A), CLL69 (6815delA) and CLL93 (5290delC). To confirm this pattern of responses, I investigated the phosphorylation of multiple downstream targets for CLLs 119 and 75 (Figure 6.15). By comparison with CLL138, which had an 11q deletion and a second wild type *ATM* allele, CLLs 119 and 75 both had impaired phosphorylation of ATM, SMC1, Nbs1 and p53 and this was particularly marked for CLL119. Indeed the responses in CLL119 were similar to those in CLL15 that had lost both functional *ATM* alleles despite the high level of expression of ATM protein and a certain degree of serine 1981 ATM phosphorylation in CLL119.

In the case of CLL 69, although the level of phosphorylation was significantly reduced relative to the control tumour, there nevertheless was evidence of some retention of low level ATM activity. The investigation of the change in p53 and p21 expression after IR confirmed that CLL 69 did indeed have a deficient response. Impairment in the up regulation of p53 and

Figure 6.14

There are deficient phosphorylation responses following DNA damage with IR (5Gy) in CLL tumours 93, 75, 119 and 69 that all have a mutation in one *ATM* allele but with no loss of the remaining *ATM* allele, in comparison to CLL tumours 61, 158, 138 and 34, respectively, which have no *ATM* mutations.

Figure 6.14

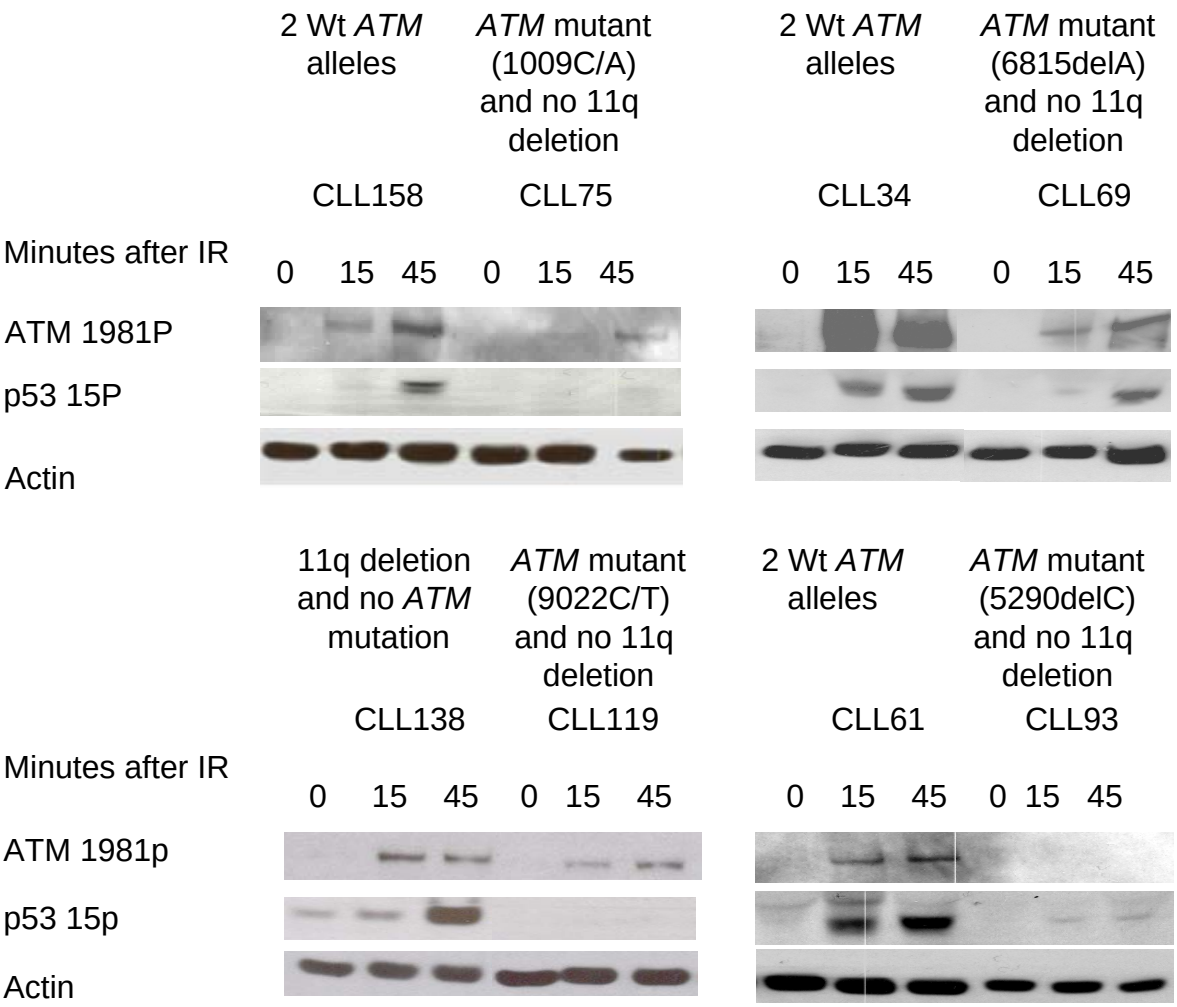
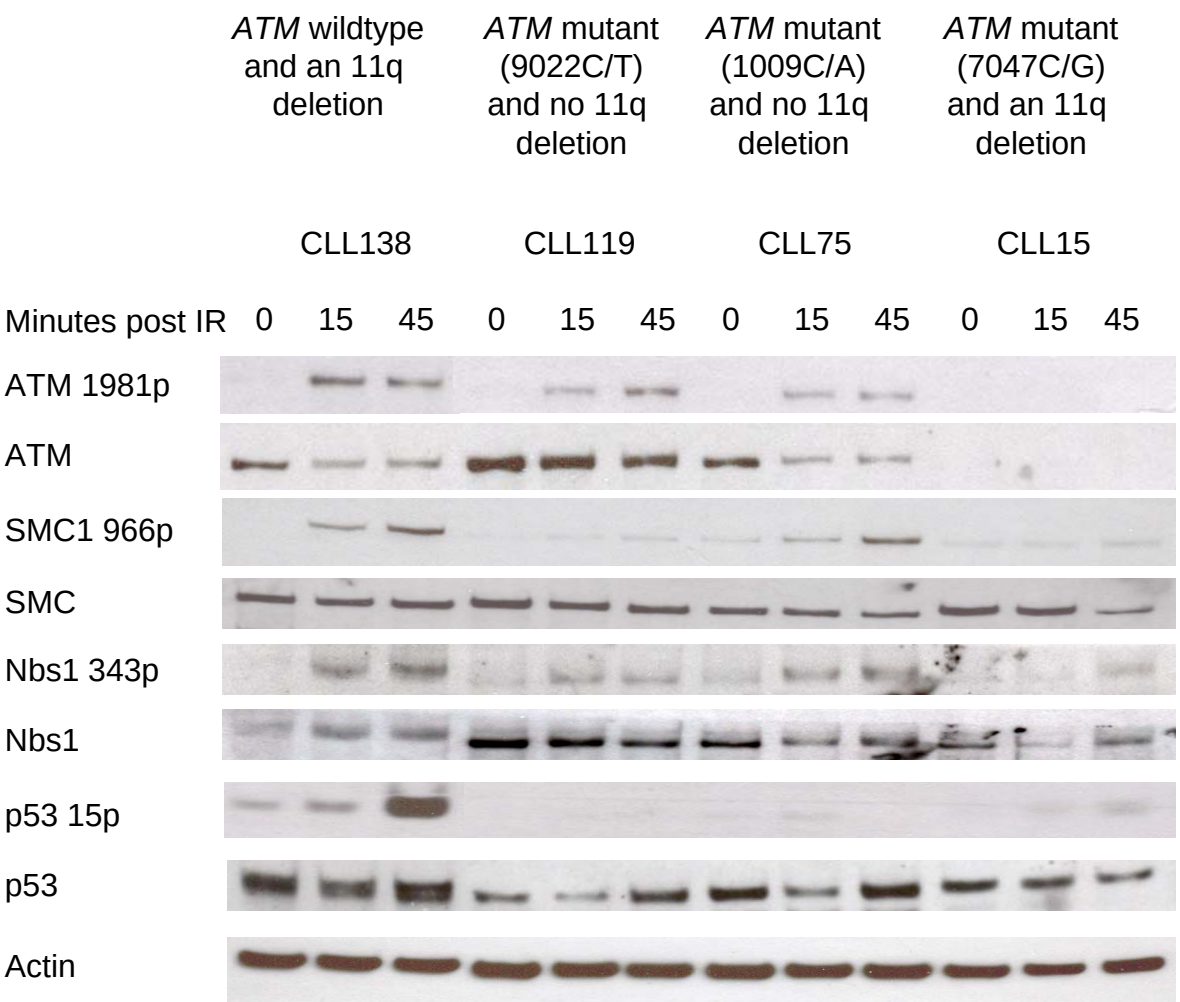

2 Wt *ATM* alleles
ATM mutant (1009C/A) and no 11q deletion
2 Wt *ATM* alleles
ATM mutant (6815delA) and no 11q deletion
CLL158
CLL75
CLL34
CLL69
Minutes after IR
0 15 45 0 15 45
0 15 45 0 15 45
ATM 1981P
p53 15P
Actin
11q deletion and no *ATM* mutation
ATM mutant (9022C/T) and no 11q deletion
2 Wt *ATM* alleles
ATM mutant (5290delC) and no 11q deletion
CLL138
CLL119
CLL61
CLL93
Minutes after IR
0 15 45 0 15 45
0 15 45 0 15 45
ATM 1981p
p53 15p
Actin

Figure 6.15

There is impairment of ATM dependent phosphorylation of mutiple targets in CLLs119 and 75 with one mutant and one wild type *ATM* allele in comparison to CLL138 with a deletion of chromosome 11q but no ATM mutation following IR (5Gy). The deficient responses in CLLs119 and 75 are comparable to the deficient response that is seen in CLL15 that has bi-allelic *ATM* defects.

Interestingly, the baseline level of ATM protein in CLL119 was higher than in CLL138 and therefore this suggested that the deficiency in the phosphorylation of downstream substrates in this tumour was due to impairment in the function rather than the amount of ATM protein.

Figure 6.15


ATM wildtype and an 11q deletion
ATM mutant (9022C/T) and no 11q deletion
ATM mutant (1009C/A) and no 11q deletion
ATM mutant (7047C/G) and an 11q deletion
CLL138
CLL119
CLL75
CLL15
Minutes post IR 0 15 45 0 15 45 0 15 45 0 15 45
ATM 1981p
ATM
SMC1 966p
SMC
Nbs1 343p
Nbs1
p53 15p
p53
Actin

induction p21 expression was also seen for CLL tumours 119 and 75 (Figure 6.16).

One explanation to account for the abnormal ATM function in these tumours is that the mutations are resulting in a dominant negative effect. In this situation, the ATM protein that is derived from the mutant *ATM* allele interferes with the activity of the wild type ATM protein. Such a mechanism could potentially apply to the mis-sense mutations 9022C/T (CLL119) and 1009C/A (CLL75), in this study, and indeed a dominant negative effect has been previously reported for several *ATM* sequence changes (Scott SP *et al*, 2002). By comparison, such a dominant negative mechanism could not explain the impairment of ATM function in the CLLs 93 and 69 that had truncating mutations. Thus, it was possible that these tumours harboured a second mutation that had escaped detection or that they had acquired a new 11q deletion.

In order to confirm that there was no 11q deletion in the tumours at the time that I performed the functional analysis, I undertook FISH studies on three of these four tumours (CLLs 69, 75 and 119), where viable cells were available. In all three cases, I was able to demonstrate the presence of two ATM probes in the tumours cells, thus confirming that there was no deletion of chromosome 11q (Figure 6.17).

In a further 3 cases [CLL 124 (5228C/T), CLL 84 (8592C/T) and CLL 92 (5980A/G)] from this genetic category with one wild type and one mutant *ATM* allele, there was some reduction in the level of phosphorylated ATM after DNA damage but normal phosphorylation of the downstream target p53. Two of these cases CLL124 and CLL84 also had a mutation in the *TP53* gene and

Figure 6.16

There is abnormal up regulation of p53 and induction of p21 expression following IR in CLLs 75, 119 and 69 with one mutant and one wild type *ATM* allele compared to CLLs 98, 24, 12 and 91 that all have with two wild type *ATM* alleles.

Figure 6.16

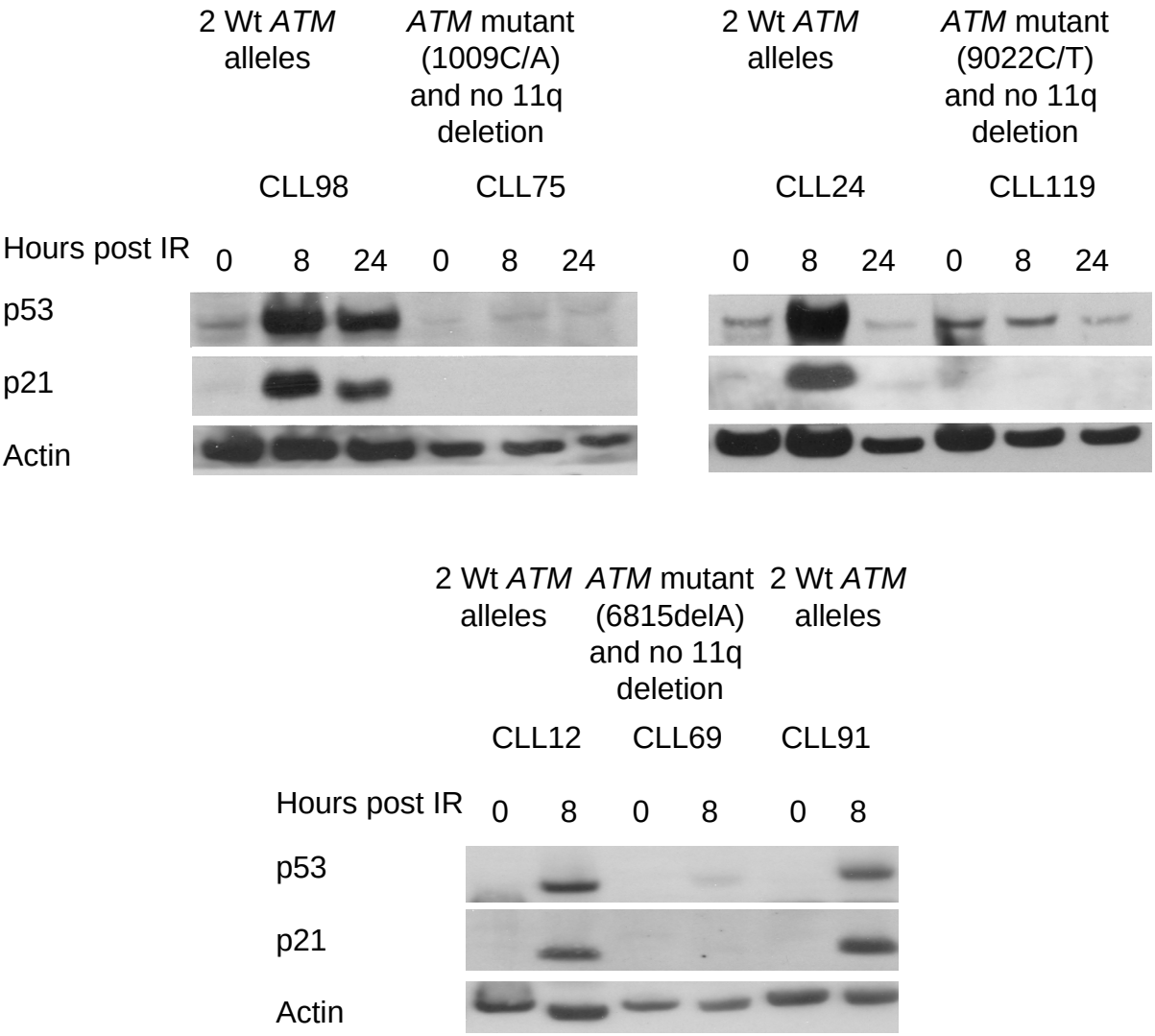

2 Wt *ATM* alleles
ATM mutant (1009C/A) and no 11q deletion
2 Wt *ATM* alleles
ATM mutant (9022C/T) and no 11q deletion
CLL98
CLL75
CLL24
CLL119
Hours post IR
0 8 24 0 8 24
0 8 24 0 8 24
p53
p21
Actin
2 Wt *ATM* alleles
ATM mutant (6815delA) and no 11q deletion
2 Wt *ATM* alleles
CLL12
CLL69
CLL91
Hours post IR
0 8 0 8 0 8
p53
p21
Actin

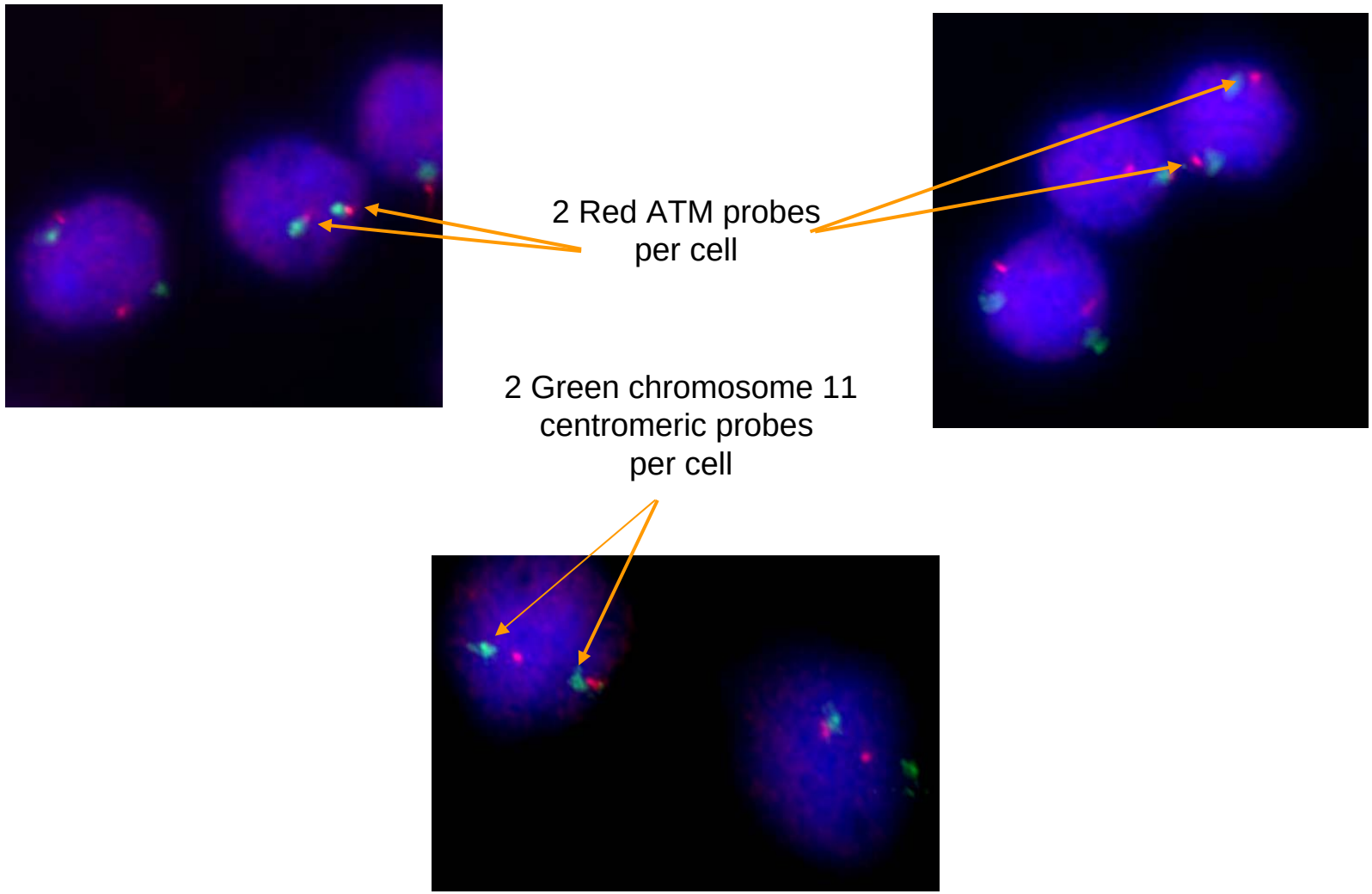
Figure 6.17

There is no evidence of a deletion of 11q in CLLs 69, 75 or 119 using FISH analysis. In all three cases there are two red ATM probes as well as two green chromosome 11 centromeric probes in all of the tumour cells,

Figure 6.17

CLL69

CLL75



CLL119

as such they had high basal levels of p53 protein, which is characteristic of tumours with mutant p53 protein (Pettitt AR *et al* 2001, Stankovic T *et al*, 2002). As a result there was actually an increased level of p53 phosphorylated on serine 15 in these tumours, compared to tumours with two wild type *ATM* alleles. The lower levels of auto-phosphorylated ATM in these tumours could indicate that, despite the preserved phosphorylation of one downstream target, the *ATM* mutation is interfering with certain specific aspects of ATM function (Figure 6.18). However, it is difficult to conclude that these tumours have an impairment of ATM activity based only on experiments that show reduced levels of ATM phosphorylation since many tumours that are heterozygous at the ATM locus, due to an 11q deletion, also have reduced levels of phosphorylated ATM after IR. Notably, however, in the case of CLL124 the defective up regulation of phosphorylated ATM did not appear to be simply due to a reduction in the level of ATM protein (Figure 6.18). In all three cases, I would ideally need to assess the phosphorylation of additional downstream targets to determine with certainty if there was defective ATM function. These experiments were not possible due to the lack of further viable cells.

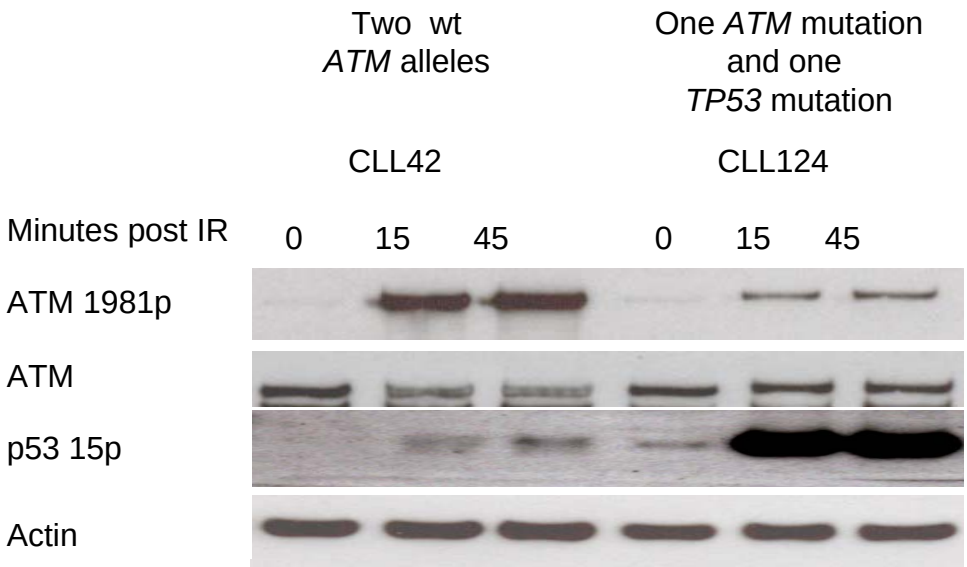
In the remaining two cases, CLL 113(3964C/A) and CLL27(3383A/G), in this subgroup, all aspects of ATM function were equivalent to tumours with two wild type *ATM* alleles, suggesting retained ATM activity (data not shown).

Altogether I have shown that the group of tumours with one mutant *ATM* allele and one wild type *ATM* allele behaved heterogeneously with respect to the cellular ATM activity in this study. From the nine cases investigated, there was deficient ATM activity in four cases, with the possibility of impairment of

Figure 6.18

There is an increased level of phosphorylated p53 on serine 15 in CLL124, which has an *ATM* and a *TP53* mutation in comparison to CLL42 with wild type *ATM* and *TP53* genes following IR (5Gy). By comparison there is a reduced level of phosphorylated ATM on serine 1981 in CLL124, despite normal levels of ATM protein. This suggests impairment in one aspect of ATM function in CLL124 as a consequence of the *ATM* mutation.

Figure 6.18


Two wt
ATM alleles
One ATM mutation
and one
TP53 mutation
CLL42
CLL124
Minutes post IR
0
15
45
0
15
45
ATM 1981p
ATM
p53 15p
Actin

the function of ATM in additional cases and retained ATM activity in at least two cases.

All the tumours with one mutant and one wild type *ATM* allele were derived from the unselected cohort. In addition to these eleven cases, there were also seven tumours from this cohort that had abnormal ATM activity as a consequence of two abnormal *ATM* alleles (Table 4.3). Therefore, the majority of tumours that had *ATM* mutations from this unselected cohort could be demonstrated to have some impairment of ATM activity and this would suggest that loss of ATM function is the mechanism for the poor clinical outcome that I observed in this group of patients.

6.4 Role of the ATM protein in the cellular response to fludarabine

Having investigated the functional consequences of *ATM* mutations in CLL tumours in the response to IR, I next assessed their impact on the response to fludarabine. Initially, I investigated the role of ATM in the response to fludarabine using primary CLL tumour cells that were known to have two wild type *ATM* alleles, and subsequently I assessed the effects of combination of mutations and deletion of *ATM* alleles on these responses.

6.4.1 Induction of DNA breaks by fludarabine

To investigate whether fludarabine leads to the induction of double strand DNA (dsDNA) breaks in CLL tumour cells, I performed immunofluoresence experiments. Following DNA damage, a γH2AX focus will form at the site of each dsDNA break and, as such, the formation of these foci is a highly specific marker for the level of dsDNA breaks in each cell. I was able to

confirm the induction of γH2AX foci in two representative CLL tumours with wild type *ATM* genes following treatment with fludarabine. These foci were detectable after 8 hours of incubation with 10μM of Fludarabine and were more obvious after 24 hours of treatment. At 24 hours the frequency and prominence of γH2AX foci were similar to that seen in CLL cells 3 hours after irradiation (Figure 6.19).

6.4.2 Induction of phosphorylation responses by fludarabine

Having demonstrated that fludarabine leads to production of DNA DSBs, I next wanted to assess whether there was evidence of induction of ATM dependent phosphorylation responses. Initially, I performed a time course experiment. In keeping with the timing of the induction of γH2AX foci, I showed that phosphorylation of both ATM and p53 could be detected after 4 and 8 hours of treatment with 20μM fludarabine but these responses were maximal after 24 hours of treatment. There was also evidence of up regulation of the total p53 levels, indicating stabilisation of the p53 protein through phosphorylation, after 4 hours treatment with fludarabine which was again further increased after 24 hours (Figure 6.20).

In order to verify that these phosphorylation responses were an effect of the fludarabine rather than a non-specific effect of cell culture, I performed an experiment where CLL cells were cultured both in the presence and absence of fludarabine for up to 48 hours. I showed that, while there was phosphorylation of ATM, SMC1 or p53 in the cells treated with fludarabine, these targets were not phosphorylated after 24 hours of culture in the

Figure 6.19

There is induction of γH2AX foci, as detected by immuno-fluorescence, following 8 and 24 hours of treatment with fludarabine in a CLL tumour. The numbers of foci within each CLL cell nuclei is greatest after 24 hours of treatment with fludarabine and at this time point the frequency of foci per cell are similar to that seen in CLL tumours cells at 3 hours after IR (5Gy).

Figure 6.19

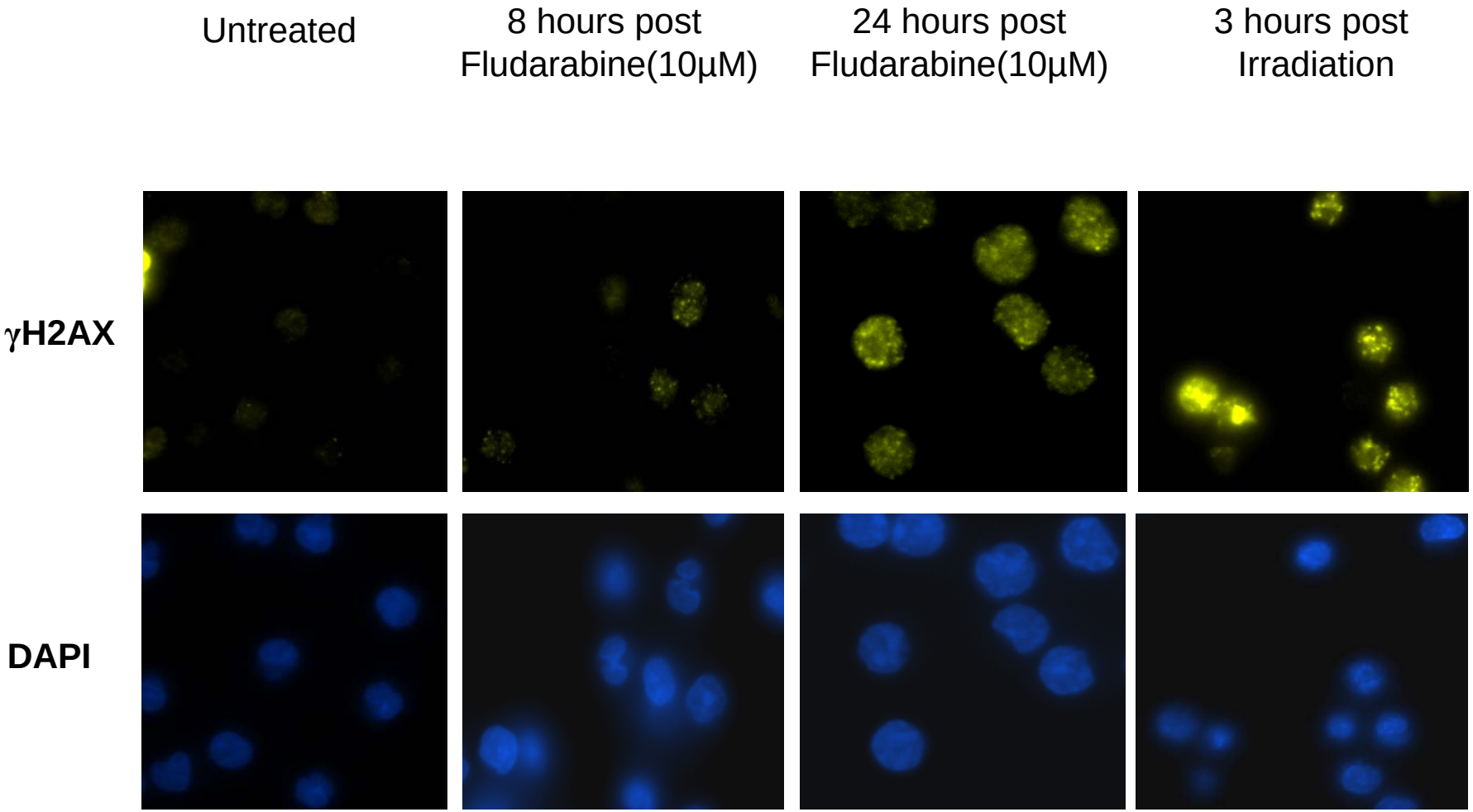
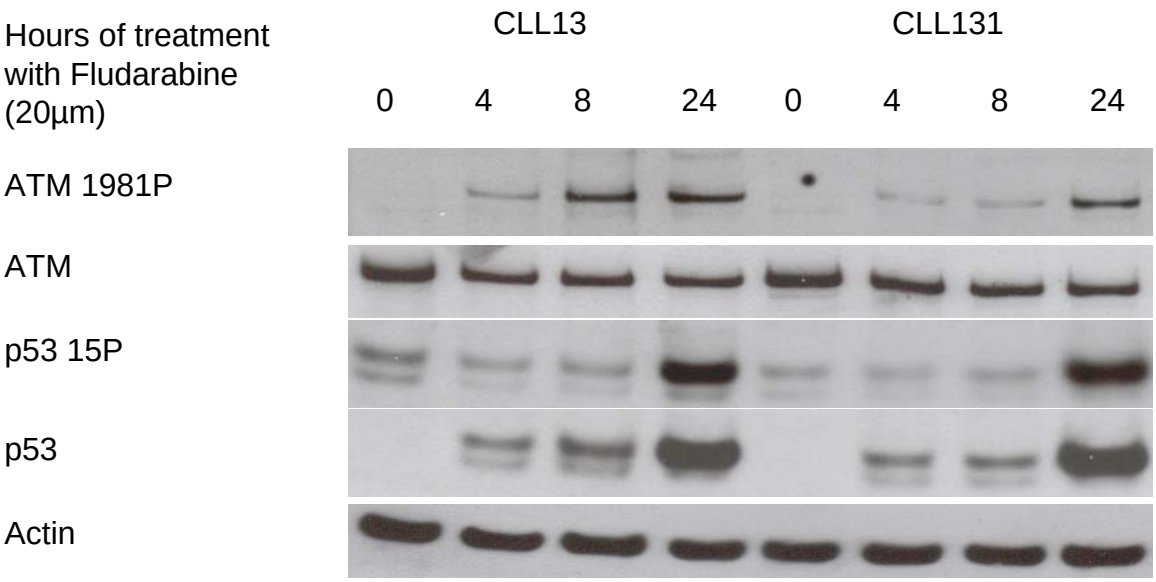

Untreated
8 hours post Fludarabine(10µM)
24 hours post Fludarabine(10µM)
3 hours post Irradiation
γH2AX
DAPI

Figure 6.20

There is phosphorylation of ATM on serine 1981 and p53 on serine 15 following treatment with fludarabine in CLLs 13 and 131 that had two wild type *ATM* alleles. This could be detected after 4 hours treatment with fludarabine but was maximal after 24 hours of treatment.

Figure 6.20


Hours of treatment with Fludarabine (20µm)
CLL13
CLL131
0
4
8
24
0
4
8
24
ATM 1981P
ATM
p53 15P
p53
Actin

absence of the drug, therefore, confirming that the responses were indeed due to fludarabine (Figure 6.21).

In addition, I also assessed the cleavage of PARP1 as an indication of apoptosis in these tumours. I was able to demonstrate that there was only evidence of induction of apoptosis in the cells that were treated with fludarabine. This was evident after at 48 hours of fludarabine treatment, whereas the phosphorylation responses were present after 24 hours of treatment. Therefore, this affirmed that the activation of this DNA damage response pathway was a direct effect of fludarabine leading to the induction of dsDNA breaks, and not a non-specific late event resulting from DNA fragmentation in association with apoptosis (Figure 6.21).

6.4.3 Effects of *ATM* mutations in the response to fludarabine

Although ATM is the principal protein in the cell that coordinates the response to dsDNA breaks there is also some redundancy between ATM and related proteins, most notably Ataxia Telangiectasia Related protein (ATR). ATM is known to be particularly important for a rapid kinetic response following DNA damage but other related proteins such as ATR can perform many of its functions but with slower kinetics (Helt CE *et al* 2005, Myers JS et al, 2006). Ionising irradiation leads to the immediate and simultaneous induction of a large number of DNA breaks whereas the kinetics of drug induced DNA damage are more complex. As I have demonstrated in the experiments above, DNA DSBs appear to accumulate over time in CLL tumours following fludarabine treatment. Therefore, there is a possibility that ATM is not an absolute requirement for the induction of the phosphorylation

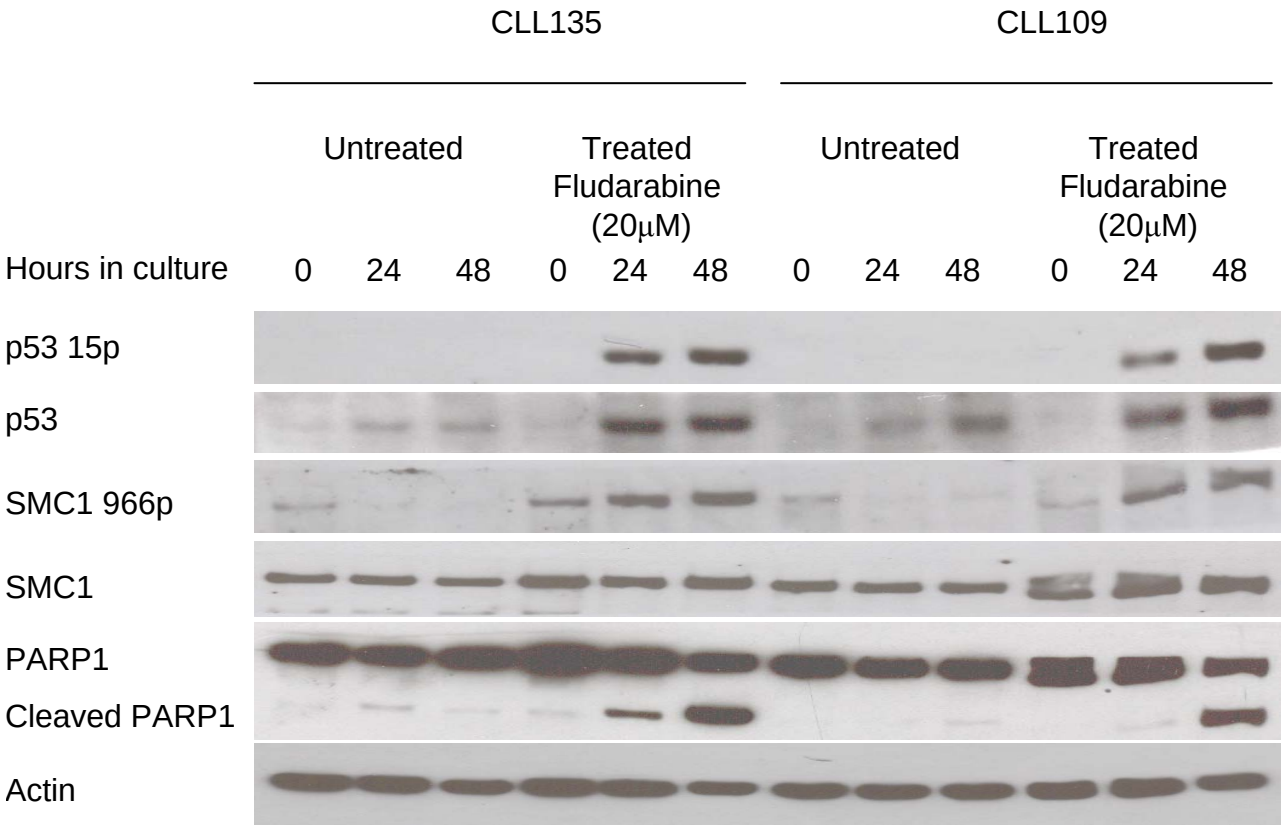
Figure 6.21

There is phosphorylation of p53 and SMC1 in CLLs 135 and 109, which have wild type *ATM*, following 24 hours of treatment with fludarabine. There is no phosphorylation of these targets in tumours cells that were cultured in the absence of drug.

Cleavage of PARP1, as an indicator of apoptosis, occurs following 48 hours of treatment with fludarabine. There is no PARP1 cleavage in the cultured cells in the absence of fludarabine, indicating an absence of spontaneous apoptosis.

Therefore, the phosphorylation responses are a direct effect of fludarabine. As these responses precede the onset of apoptosis, this indicates that fludarabine must be directly activating the DNA damage response pathway rather than the activation of this pathway occurring as a late event in association with apoptosis and non-specific DNA fragmentation.

Figure 6.21


CLL135
CLL109
Untreated
Treated Fludarabine (20μM)
Untreated
Treated Fludarabine (20μM)
Hours in culture
0 24 48 0 24 48 0 24 48 0 24 48
p53 15p
p53
SMC1 966p
SMC1
PARP1
Cleaved PARP1
Actin

responses that I had observed in the CLL tumours after fludarabine treatment. To clarify the specific role of ATM in the response to fludarabine, I investigated the activity in this pathway in tumours with *ATM* mutations that had been previously shown to have deficient responses to DNA damage with IR [Figures 6.3 (CLL152), Figure 6.4 (CLL11)].

I demonstrated defective phosphorylation of the ATM targets including ATM on serine 1981, SMC1 on serine 966, p53 on serine 15 and Nbs1 serine on 343, following treatment with fludarabine in the 2 tumours (CLLs152 and 11) with abnormal ATM function, consequent of bi-allelic *ATM* defects, in comparison to the proficient phosphorylation of these targets in 5 CLL tumours (CLLs 135, 32, 13, 131 and 109) with two wild type *ATM* alleles (Figure 6.22).

I next wanted to investigate whether there were differential responses, similar to those I had observed with IR, between the 11q deleted tumours with a second wild type *ATM* allele compared to those with a second mutant *ATM* allele. I studied the responses in 4 tumours with an 11q deletion and no mutation in the second *ATM* allele [CLLs 138 (80% of cells with an 11q deletion), 37 (75%), 25 (97%), 65 (91%)] and 4 tumours with both an 11q deletion and an *ATM* mutation [CLLs 07 (92% of cells with an 11q deletion), 169 (100%), 152 (99%), 57 (90%)]. The tumours with loss of an *ATM* allele but with a remaining wild type allele demonstrated ATM phosphorylation of targets following fludarabine treatment that was equivalent to that seen in tumours with two wild type *ATM* alleles. However, the CLLs with an 11q deletion and a mutant *ATM* allele all demonstrated deficient responses to fludarabine (Figure 6.23).

Figure 6.22

There is deficient phosphorylation of ATM, SMC1, Nbs1 and p53 following treatment with fludarabine in CLL152 and 11, which have bi-allelic *ATM* defects, in comparison to the responses in CLLs 135, 32, 13, 131 and 109, which have 2 wild type *ATM* alleles.

Figure 6.22

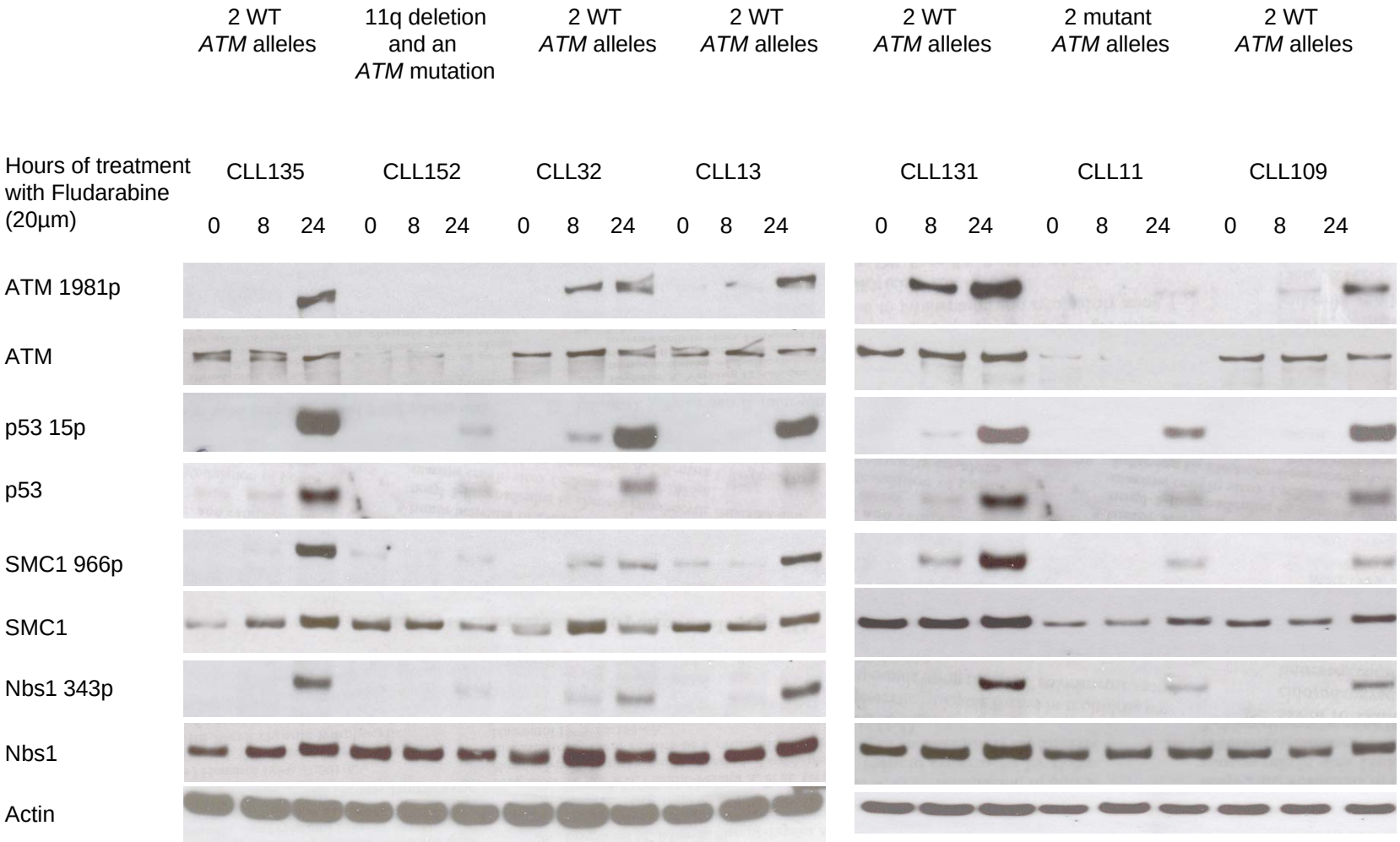
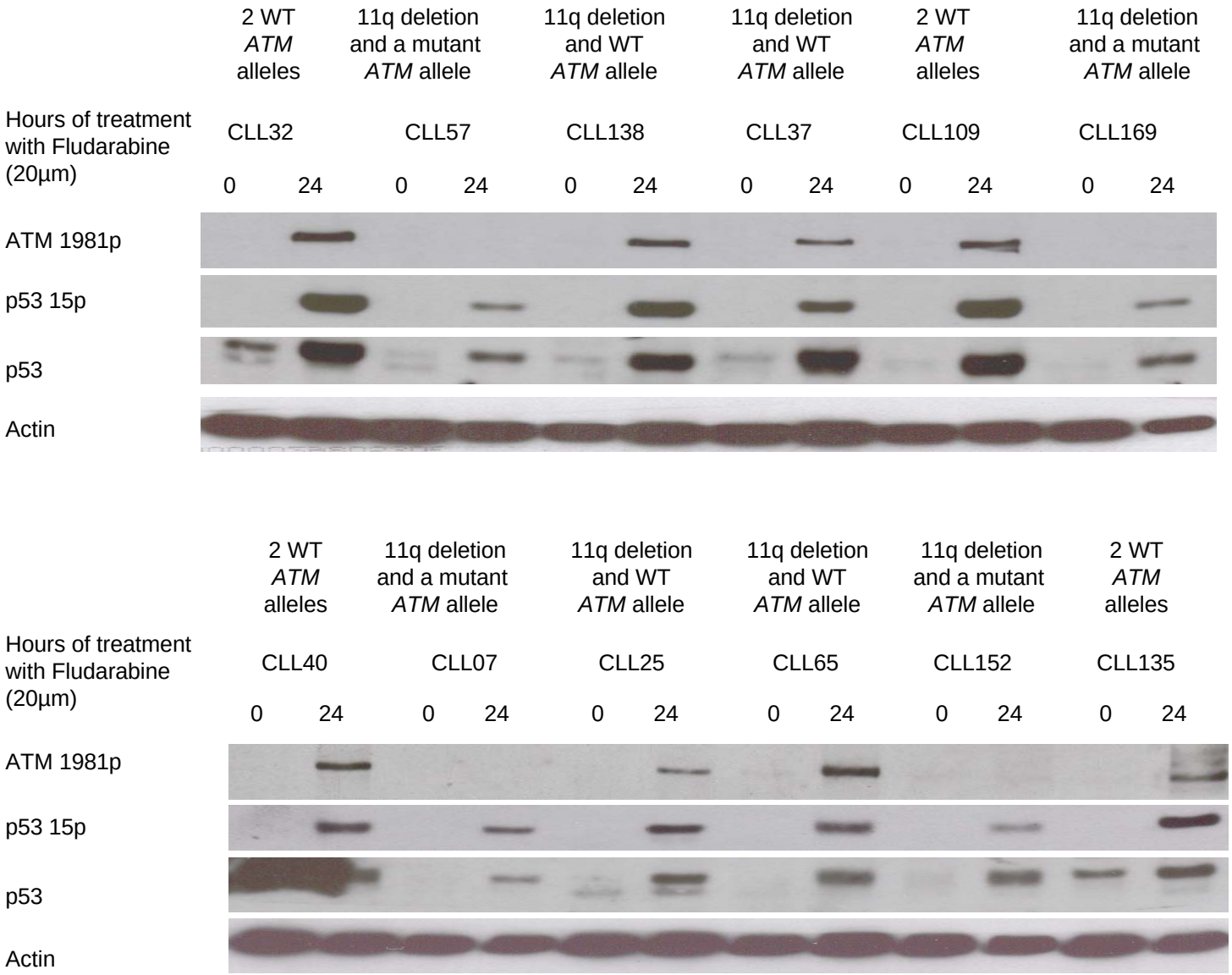

2 WT *ATM* alleles
11q deletion and an *ATM* mutation
2 WT *ATM* alleles
2 WT *ATM* alleles
2 WT *ATM* alleles
2 mutant *ATM* alleles
2 WT *ATM* alleles
Hours of treatment with Fludarabine (20µm)
CLL135
CLL152
CLL32
CLL13
CLL131
CLL11
CLL109
0 8 24 0 8 24 0 8 24 0 8 24 0 8 24 0 8 24 0 8 24
ATM 1981p
ATM
p53 15p
p53
SMC1 966p
SMC1
Nbs1 343p
Nbs1
Actin

Figure 6.23

There are differential responses to fludarabine according to the status of the remaining *ATM* gene in CLL tumours with an 11q deletion. CLLs 57, 169, 07 and 152, with an 11q deletion and a mutant *ATM* allele, have impaired phosphorylation of both ATM and p53 following treatment with fludarabine at 24 hours. In contrast, CLLs 138, 37, 25 and 65, with an 11q deletion but a second wild type *ATM* allele, have normal phosphorylation responses which are equivalent to CLLs 32, 109, 40 and 135 that have two wild type *ATM* alleles.

Figure 6.23


2 WT *ATM* alleles
11q deletion and a mutant *ATM* allele
11q deletion and WT *ATM* allele
11q deletion and WT *ATM* allele
2 WT *ATM* alleles
11q deletion and a mutant *ATM* allele
Hours of treatment with Fludarabine (20µm)
CLL32
CLL57
CLL138
CLL37
CLL109
CLL169
0 24 0 24 0 24 0 24 0 24 0 24
ATM 1981p
p53 15p
p53
Actin
2 WT *ATM* alleles
11q deletion and a mutant *ATM* allele
11q deletion and WT *ATM* allele
11q deletion and WT *ATM* allele
11q deletion and a mutant *ATM* allele
2 WT *ATM* alleles
Hours of treatment with Fludarabine (20µm)
CLL40
CLL07
CLL25
CLL65
CLL152
CLL135
0 24 0 24 0 24 0 24 0 24 0 24
ATM 1981p
p53 15p
p53
Actin

These results, therefore, provide direct evidence for the role of ATM in the response to fludarabine, and indicate that proteins such as ATR cannot fully compensate for the function of ATM in CLL tumours with ATM deficiency. They also provide further evidence to suggest that CLL tumours with an 11q deletion form functional subgroups according the mutation status of the *ATM* allele and that this impacts on the responses to DNA damaging chemotherapeutic drugs as well as irradiation.

6.5 Conclusions

In this chapter, I have demonstrated that those CLL tumours with two or more *ATM* genetic defects had impairment of ATM activity. It should therefore be possible to identify these tumours using functional measurements of ATM activity and I have shown that measurement of the early phosphorylation responses after DNA damage with irradiation was the most specific assay. I have also shown that *ATM* mutations have an impact on the downstream p53-dependent apoptotic pathway, therefore validating the pathogenic nature of the mutations, which I had identified.

The functional consequences of an abnormality of a single *ATM* allele are less clear. The subgroup of tumours with one mutant allele and one wild type *ATM* allele behaved heterogeneously with respect to the responses to DNA damage. While several tumours had a deficient response, the response in other tumours was either normal or possibly partially deficient. In contrast, the subgroup of tumours with loss of one *ATM* allele through an 11q deletion but with a second wild type *ATM* allele behaved more homogeneously and had

retained ATM activity that was similar to that seen in tumours with two wild type *ATM* alleles.

The mechanism of action of many drugs used in treatment of CLL involves DNA damage. In this study I have shown that the chemotherapeutic drug, fludarabine induces DNA DSBs and leads to the induction of ATM dependent phosphorylation responses. In comparison to the tumours with two wild type *ATM* alleles, I observed impairment in these responses in those tumours with an *ATM* mutation and an 11q deletion but proficient responses in tumours with one retained wild type *ATM* allele.

Discussion

The purpose of my project has been to study the role of *ATM* mutations in the pathogenesis of chronic lymphocytic leukaemia. I have examined the prevalence of *ATM* mutations, their nature and distribution across the gene, the timing of their occurrence and both their clinical consequences in CLL patients and cellular consequences in CLL tumours. I would like to discuss my results by considering the value of *ATM* mutations as a prognostic marker in CLL patients and the implications of *ATM* mutations in the biology of CLL tumours.

7.1 *ATM* mutations as a prognostic marker in CLL

Due to the marked clinical heterogeneity of CLL, an important focus of the research in this disease has been to identify biological markers that can both be useful in the clinical setting and also lead to an increase in the understanding regarding the nature of CLL.

In order for *ATM* mutations, or any other candidate parameter, to be potentially useful as a prognostic marker in CLL, they should ideally:

a) Have a significant prevalence in CLL

b) Impact on clinical outcome

c) Occur early in the course of the disease

d) Provide independent prognostic information

e) Be readily detectable

f) Provide an insight into the biology of CLL

g) Guide therapeutic decisions

7.1.1 Prevalence of *ATM* mutations in CLL patients

I found *ATM* mutations in 18 out of 155 unselected CLL patients, which corresponds to a prevalence of 12%. This is lower than has been found in previous smaller cohorts. However, whereas previous studies have included more patients with aggressive disease features, my cohort was characterised by a high proportion of individuals with indolent disease (Stankovic T *et al*, 2002, Schaffner C *et al*, 1999). For example, there were 70% of patients with mutated *IGVH* genes and just 20% had advanced stage B or C disease at diagnosis in my study.

My aim had been to establish a representative spectrum of CLL patients that is seen in haematology departments in order to investigate the proportion of the patients that had *ATM* mutations. The type of cohort that I established in this project will include more patients with indolent disease than a cohort that is based on the identification and follow up of all patients diagnosed with CLL. This is because in my cohort, which included all patients that currently had CLL at a given time period, more patients with indolent disease than with aggressive disease will have survived in order to be included in the cohort. As a result, if the mutations have a detrimental effect on survival, then the prevalence of *ATM* mutations in a group of CLL patients at any one time will be lower than the frequency of CLL patients that actually develop *ATM* mutations.

The number of mutations detected will also be affected by the sensitivity of the analysis method that is utilised. I used DHPLC for mutation screening and this method has been previously reported to have a sensitivity that is as high as 90% for the detection of mis-sense changes or small deletions and

insertions in the *ATM* gene (Bernstein JL *et al*, 2003). However, to achieve such a high sensitivity requires the analysis of certain exons with complex denaturing profiles at more than one temperature. In this study, due both to the large size of the *ATM* gene and the large number of CLL and control cases that were analysed, a compromise temperature for each exon was chosen and this may have led to a reduction in the sensitivity for mutation detection for certain *ATM* exons. However, I was able to reliably detect the common polymorphisms that are known to occur in the *ATM* gene, and I also found a large number of less common polymorphic variants suggesting that the DHPLC method was indeed detecting the vast majority sequence changes that were actually present in the *ATM* gene in this study.

Mutation detection by DHPLC relies on the development of heteroduplexes and thus requires the presence of both alleles of a gene. Therefore, in all cases that were known to have an 11q deletion I performed mixing experiments as outlined in the methods. However, since the 11q status was not known for all the tumours in the cohort, this could also have potentially resulted in a failure to detect a mutation that was in fact present. Nevertheless, I do not think that this had a major effect on the sensitivity of mutation detection in this cohort. Given the known prevalence of 11q deletions in CLL, it is unlikely that there would have been a significant number of tumours with an unknown chromosome 11q deletion. Furthermore, heteroduplexes were still detected in the majority of tumours that were known to have an 11q deletion even in the absence of mixing experiments. Previously, it has been shown that only 5% of the cells present need to have two alleles in order to allow heteroduplex detection by DHPLC (Xiao W,

Oefner PJ, 2001). Notably, in CLL tumours that have an 11q deletion there is frequently a small sub-clone that retains both intact chromosomes, as presented in the results of this study (Dewald GW *et al*, 2003). Finally, the ability to detect an *ATM* polymorphism in any sample indicates that it was possible to form heteroduplexes and, therefore, it should also be possible to detect a mutation. In this study one or more *ATM* polymorphisms were detected in 107 patients from the cohort 155 of patients.

Thus, in summary, I found that *ATM* mutations do indeed affect a significant proportion of all CLL patients. My results indicate that within an unselected population of CLL patients, approximately 12% of patients are likely to carry an *ATM* mutation at any point in time. This would therefore represent the commonest single genetic defect to be identified in CLL patients. However, as discussed above, the proportion of the total population of patients diagnosed with CLL that acquire an *ATM* mutation at some time in their disease is almost certainly higher than this prevalence of patients with an *ATM* mutation at a given time point. This is clearly an important consideration when determining the prospective value of a biological marker.

7.1.2 The impact of *ATM* mutations on clinical outcome

The most fundamental characteristic of a prognostic marker is its ability to predict outcome. The consequences on clinical outcome of *ATM* mutations in CLL patients have not been previously assessed. I analysed the effects of *ATM* mutations on the overall survival (OS) of CLL patients from diagnosis and on the time for requirement of treatment, namely, the treatment free survival (TFS).

Outside the context of a clinical trial, the OS and TFS from diagnosis are established ways to assess the effects of a biomarker on clinical outcome and have been previously used to validate various prognostic markers in CLL, such as *IGVH* mutation status, Zap-70 expression and cytogenetic abnotmalities (Damle RN *et al* 1999, Hamblin T *et a*l 1999, Dohner H *et al* 1999, Wiestner A *et al*, 2003). Parameters such as the attainment or persistence of complete or partial remissions are more difficult to assess outside a trial because CLL patients will typically have received a wide variety of different treatment schedules (Oscier D *et al*, 2004). OS and TFS reflect different aspects of disease biology and ideally a prognostic marker will impact on both parameters.

OS from diagnosis encompasses both the need for treatment together with the degree and persistence of any initial and subsequent treatment responses. However, not all deaths in CLL patients will be related to their underlying disease due to the high median age of onset of this disease. In addition, the distinction between leukaemia-related deaths and unrelated deaths is not always straightforward because immune dysregulation can increase the risk of infection, auto-immune disease and also secondary malignancies (Oscier D *et al*, 2004). However, any non-leukaemia related deaths would be expected to occur in equal proportions in all subgroups.

TFS reflects the extent and severity of the disease because only CLL patients that have a significant disease burden leading to clinical symptoms will receive treatment. However, TFS clearly does not encompass either the degree of response to treatment or the persistence of that response. It is also less objective than OS because there may be inter-physician variation in the

threshold for initiating treatment and the decision to commence treatment might be influenced by factors such as patient age and co-morbidity, in addition to the severity of the CLL (Oscier D *et al*, 2004).

This study was largely a retrospective study, where the tumours samples were collected and analysed for *ATM* mutations over a given time period but survival parameters were measured from the time of diagnosis to the time of either death or treatment or, if these had not occurred, to the time of the data analysis. Such a retrospective approach has been used to evaluate the impact of several prognostic markers in CLL and although a prospective study would be advantageous, it would take many years to accumulate data due to the long median survival of CLL patients (Damle RN *et al* 1999, Hamblin T *et a*l 1999, Dohner H *et al* 1999, Wiestner A *et al*, 2003). A retrospective approach assumes that the biomarker that is being evaluated was in fact present at the time of diagnosis and is stable over time. Furthermore, as discussed above, in retrospective studies there is the potential for the impact of a marker to be influenced by the fact that more patients with indolent disease will survive to be included in a cohort.

By the approach taken in this study I was able to show that patients with *ATM* mutations had a statistically significant reduction in both treatment free survival and overall survival compared to patients that retained wild type *ATM* genes. This is a novel finding and provides key clinical evidence to support the hypothesis that the loss of function of ATM is important in influencing the behaviour of the CLL tumour cells. Since *ATM* mutations had a clear impact on both parameters, this indicates that *ATM* mutations influence the behaviour of the tumour before treatment as well as the extent of treatment responses.

The timing of *ATM* mutations is discussed below, but one would assume that any mutations developing after diagnosis would lead to an under- rather than over-estimation of their effect on a parameter that is measured from the time of diagnosis. To investigate the effect of the retrospective nature of my cohort on the clinical consequences of the *ATM* mutations I included the 'era of diagnosis' as a parameter in the multivariate analysis. By this method, I was able to confirm that the retrospective nature of the cohort had resulted in an underestimation of the impact of *ATM* mutations on OS and that the true effect might be even higher.

7.1.3 The timing of *ATM* mutations

In order to have value as a biomarker the development of an *ATM* mutation would ideally have to be an early event in the pathogenesis of CLL and would have to pre-date the clinical signs of an aggressive phenotype such as advanced stage or rapid lymphocyte doubling time.

I investigated the timing of the development of *ATM* mutations in this study by assessing germ-line material and also by performing a 'look back' analysis in those tumours where material was available that had been collected at earlier times during the disease course. Altogether, germ-line mutations were detected in 5 CLL patients, 3 from the original unselected cohort and 2 from the cohort of 11q-deleted tumours. Clearly these mutations, by definition, would be present at the time of diagnosis of the disease. As part of the look back analysis, I found that 7 out of the 9 acquired *ATM* mutations assessed were present in the earlier sample, which in most cases had been collected around the time of diagnosis. However, two of the acquired

mutations could be demonstrated to have occurred at a time after diagnosis and, notably, in both cases this late development of an *ATM* mutation occurred in tumours that also had an 11q deletion.

Thus, these results indicate that *ATM* mutations are often early events in the pathogenesis of CLL suggesting they would indeed pre-date clinical signs of more rapid disease progression and have predictive value. However, this is not exclusively the situation and in certain tumours, possibly more commonly in those that already have an 11q deletion, *ATM* mutations can occur at a later stage during disease progression. Interestingly, both 17p and 11q deletions have also been reported to occur at stages after the initial diagnosis and these markers are established prognostic markers in CLL, suggesting that there is still predictive value in detecting these changes because they may herald a change in disease behaviour (Cuneo A *et al*, 2002, Chevalier P *et al*, 2002). The relationship between the timing of development of *ATM* mutations compared to 11q deletions is discussed later.

7.1.4 Association with existing prognostic markers

ATM mutations could impact adversely on survival due to their association with other poor prognostic factors such as age, stage, *IGVH* mutation status, *TP53* mutations, CD38 expression, Zap-70 expression and cytogenetic abnormalities. Ideally, in order to have novel value as a prognostic marker, they should provide information that is independent of existing markers.

In this study there was no association between *ATM* mutations and either age or clinical disease stage at diagnosis, and the prognostic value of *ATM*

mutations was not affected by either of these parameters in the multivariate analysis.

Previously, *ATM* mutations had been found to occur exclusively in CLL tumours with unmutated *IGVH* genes (Stankovic T *et al*, 2002). In this study, there was a positive association between *ATM* mutations and unmutated *IGVH* genes, but I also identified tumours with mutated *IGVH* genes that had *ATM* mutations. Furthermore, by using Cox regression multivariate analysis, I was able to demonstrate that *ATM* mutations still had an adverse prognostic effect regardless of *IGVH* mutation status, therefore indicating that they were providing independent prognostic information. One explanation to account for this finding is that tumours with *IGVH* unmutated genes may be more likely to acquire *ATM* mutations but that once an *ATM* mutation has occurred in a CLL tumour it exerts a detrimental effect regardless of the *IGVH* mutation status of that tumour.

The presence of *ATM* and *TP53* mutations in the same tumour had not been previously reported in CLL tumours, suggesting that these genetic defects were occurring in distinct CLL subgroups. In this cohort of 155 tumours I found that two of the eighteen tumours with *ATM* mutations also had mutations the *TP53* gene. In addition, I found *TP53* mutations in a further 4 independent tumours. When I compared OS and TFS between the three genetic subgroups, namely *ATM* mutant, *TP53* mutant and *ATM/TP53* wild type, I was able to show that the differences in both OS and TFS between the three groups were highly statistically significant for both parameters. For this comparison the two tumours with *ATM* and *TP53* mutations were included in the *TP53* mutant subgroup and therefore these results indicate that *ATM*

mutations are identifying a subgroup of CLL tumours with a poor outcome that is independent of the presence of *TP53* mutations. The OS in patients with *ATM* mutations could be shown to be intermediate between patients with *TP53* mutations and patients with wild type *ATM* and *TP53* genes, while the TFS survival for *ATM* mutant patients was similar to that of patients with *TP53* mutations.

The relationship between *ATM* mutations and Zap70 status and CD38 status was not specifically addressed in this study, and this warrants investigation in the future.

Thus, I demonstrated that *ATM* mutations provide prognostic information that is independent of age, clinical stage, *IGVH* mutation status and *TP53* mutation status in CLL tumours. These results therefore indicate that *ATM* mutations are identifying a subgroup of CLL tumours that have a poor outcome as a direct consequence of the mutation.

7.1.5 Relationship between *ATM* mutations and 11q deletions

The relationship between CLL tumours with *ATM* mutations and those with a deletion of chromosome 11q is of interest since all tumours found to have an 11q deletion will have loss of one *ATM* allele as it is an ATM probe that is used to detect the deletion. Therefore, if the *ATM* gene behaved as a classic tumour suppressor gene, it might be expected that there would be a close correlation between those CLL tumours with an 11q deletion and those with *ATM* mutations. Evidently if both were markers were identifying the same CLL tumour subgroup then there would be no additional prognostic value in

assessing the mutation status of the *ATM* gene over and above identifying tumours with 11q deletions.

In the cohort of 155 unselected CLL patients only four tumours that had *ATM* mutations also had a chromosome 11q-deletion. In addition there were also five tumours that had been found to have an 11q-deletion but where no *ATM* mutation was identified. However, since the 11q status was not known on all the tumours from this cohort it was not possible to perform statistical analysis, such as that used with the *IGVH* status, to assess if *ATM* mutations provide prognostic data that is independent of 11q-deletions in CLL.

Therefore, in order to further investigate the relationship between *ATM* mutations and 11q deletions I studied a second cohort of 53 tumours that all had a chromosome 11q-deletion. I detected *ATM* mutations in 20 of these tumours which corresponds to a prevalence of *ATM* mutations in CLL tumours with an 11q deletion of 39%. This result indicates that *ATM* mutations are around three fold more common in CLLs that have an 11q deletion than in unselected CLL tumours. This prevalence of 39% is higher than previously found in a smaller study where *ATM* mutations were identified in 5 of 22 tumours with an 11q deletion, and this probably reflects the fact that in the previous study mutation analysis was restricted to a 'C' terminal portion of the *ATM* coding sequence (Schaffner C *et al*, 1999). Nevertheless, this study corroborates my own results in suggesting that many 11q-deleted tumours do not have a mutation in the second *ATM* allele. These results, together with the findings in this study which show that a majority of tumours with *ATM* mutations do not have an 11q deletion, demonstrates that there is only a partial correlation between those CLL tumours with an 11q-deletion and those

with an *ATM* mutation. Thus, although there is an association between these two genetic defects, an 11q deletion is not a surrogate marker for the presence of an *ATM* mutation.

Since not all of 11q-deleted tumours have an *ATM* mutation I was interested to know if *ATM* mutations could stratify these tumours into two subgroups. In other words, could the presence of an *ATM* mutation provide additional prognostic information in this group of patients that are already known to have a poor outcome? Therefore, I analysed OS and TFS in the second cohort, where all patients had a deletion of chromosome 11q. Interestingly, there was a trend for a poorer overall survival, which approached significance, in those patients that had an *ATM* mutation in addition to an 11q-deletion compared to those that had a remaining wild type *ATM* allele. However, there was no significant difference in TFS between the two subgroups, although this was also slightly shorter in the patients with an additional *ATM* mutation in this study.

There are number of explanations to account for these results. Firstly, there may be a genuinely worse outcome in the patients with an 11q deletion and an additional *ATM* mutation, compared to those with a second wild type *ATM* allele, but because this difference is a relatively small it would require a study of a larger size to confirm. Alternatively, there may be no difference between the subgroups, suggesting that the effect of ATM function is not the principal factor that influences clinical outcome in these patients with an 11q deletion. As discussed later, the poor outcome in these patients could be related to the loss of other genes on chromosome 11q since the minimally deleted region, which has been defined, spans from 11q22.3 to q23.1 and

contains a large number of genes (Stilgenbauer S *et al*, 2006). Finally, it is possible that these two subgroups are not entirely stable over time. Thus, certain patients whose tumours have an 11q deletion could acquire an *ATM* mutation during the course of the disease and this would make longitudinal comparisons of survival measured from diagnosis between the two 'subgroups' difficult to interpret. The timing of *ATM* mutations and their functional consequences in this group of 11q-deleted CLL tumours are considered later in this discussion.

The possibility that *ATM* mutations could stratify clinical outcome in patients with 11q deletions is an interesting and potentially important question with translational implications. One way to address these issues would be to analyse a more homogeneous group of patients with an 11q-deletion in their tumour cells. For example, this could involve the *ATM* mutation analysis of samples that had been collected at the time of diagnosis, which were found to have a deletion of chromosome 11q, with subsequent follow up of outcome. Although interesting, such a study would take time to accumulate data. An alternative approach would be the analysis of the *ATM* gene in a group of 11q-deleted tumour samples that had all been collected prior to the commencement of the first treatment, ideally that were part of a clinical trial. This would then allow the assessment between the mutation status of the remaining *ATM* allele and the degree of treatment response in patients with an 11q deletion. Furthermore, the analysis of trial samples would ensure a uniformity of treatment schedules between patient groups and the effect of *ATM* status could also be correlated to efficacy of the different treatment arms.

Thus, I have shown that *ATM* mutations are associated with 11q-deleted tumours, and occur three times more commonly in this subgroup of CLL tumours than in unselected CLL tumours. However, there is only a partial correlation between those tumours with an 11q deletion and those tumours with *ATM* mutations. In addition, my results indicate that *ATM* mutations might clinically stratify the group of patients with 11q deletions and this will be a very interesting area to study in the future.

7.1.6 Identification of *ATM* mutant tumours by functional analysis

Prognostic markers that will have value in the clinical setting need to be readily detectable. *ATM* is a very large gene and therefore mutation screening is expensive and time-consuming. In addition the mutations detected in this analysis were distributed across the whole gene with only minimal clustering of the missense changes towards the 'C' terminus of the gene. This indicates that restricting analysis to just that section of the ATM gene, as previously reported, will significantly reduce the sensitivity of detection (Schaffner C *et al*, 1999). It is also unlikely that simple PCR-based tests, such as that developed to detect the V167F mis-sense mutation in the *JAK2* gene in myeloproliferative disorders, can be developed for the *ATM* gene because the mutations in *ATM* are not characterised by one or several recurrent changes (Kralovics R *et al*, 2005). Therefore, widespread mutation screening of the *ATM* gene in CLL tumours may not be possible outside the setting of a research laboratory. Instead, functional assays that reliably identify those tumours harbouring *ATM* mutations might have greater clinical potential.

The most specific role of the ATM protein in the cell is the very early response to DNA damage in the form of DSBs and ATM signals this damage by directly catalysing the phosphorylation of serine or theronine residues on numerous target substrates (Kurz EU, Lees-Miller SP, 2004). Therefore, it is likely that the assessment of these responses will most reliably reflect ATM function. One of the target substrates for ATM in this pathway is the p53 protein and as a consequence of ATM-dependent phosphorylation the level of p53 in the nucleus is up regulated (Meek D, 2004). The effects of both *ATM* and *TP53* mutations in CLL tumours have been previously investigated by assessing the levels of p53 and its transcriptional target, p21, following DNA damage with ionising irradiation. Recently, however, it has been shown that a polymorphism in the p21 protein can also influence its own level of expression in CLL tumours after IR, potentially making this functional test less useful for the classification of CLL tumours according to their underlying *ATM* status (Pettitt AR *et al* 2001, Carter A *et al*, 2006).

In this project I evaluated the effects of *ATM* mutations on the early phosphorylation of substrates after DNA damage with IR. I demonstrated that in tumours with wild type *ATM* there was phosphorylation of a range of downstream substrates that could be detected by 15 minutes after irradiation and were further increased by 45 minutes. By comparison, all of the tumours with bi-allelic *ATM* defects, which might be predicted to have impaired ATM function, had deficient phosphorylation of these downstream substrates at these time-points in this study.

I compared these early phosphorylation responses with the later changes in p53 and p21 levels after DNA damage with IR. Amongst the tumours with

bi-allelic *ATM* defects, there was a good correlation between these two assays. Interestingly, however, several of the tumours in which no *ATM* mutations had been detected exhibited impairment in the up regulation of p53 and failed to induce p21 expression. In three of these tumours I also assessed the early phosphorylation responses and in all three cases the ATM dependent phosphorylation of substrates was preserved. This suggested that the early ATM dependent phosphorylation responses might be a better reflection of ATM activity than the downstream changes in p53 and p21 levels.

Thus, I have demonstrated that measurement of the early ATM dependent phosphorylation of substrates after DNA damage with irradiation is a reliable method to assess ATM function in CLL tumours and that this assay might be more specific for the presence of *ATM* genetic defects than the later changes in p53 and p21 levels. I found that there was a good correlation between the phosphorylation of different target substrates following IR. I initially compared the phosphorylation of ATM and p53 and subsequently also studied the targets SMC1 and Nbs1. Generally my results indicated that the use of two downstream substrates such as SMC1 and p53 were the most reliable in order to assess ATM activity. This was partly due to the efficacy of the antibodies against these proteins but in addition because the assessment of the level of phosphorylated ATM after DNA damage will reflect the level of ATM protein as well as its activity. I also found that the reliability and objectivity of these assays was improved by expressing the level of each phosphorylated protein in relation to the total level of the respective protein at each time point, therefore controlling for any differences in the baseline level of these target substrates between tumours.

In all these experiments I have measured the changes in the expression of protein and phosphorylated protein using Western blotting. Interestingly, a recent publication assessed the changes in p53 and p21 levels after DNA damage using a FACS approach in order to classify CLL tumours (Carter A *et al*, 2004). This may be more convenient in a clinical diagnostic laboratory and it is possible that a similar FACS assay could be developed to measure the ATM dependent phosphorylation of substrates. This might have improved specificity and would also have the advantage of a much shorter time course for the assessment of the response to DNA damage. Furthermore, the assay could also be adapted to use agents such as etoposide or neocarcinostatin to induce the DNA DSBs, which would avoid the requirement of irradiation (D Oscier - unpublished data).

Such a functional assay could provide a means of routinely testing CLL tumours for deficient ATM function in a prospective manner. Although there are practical advantages for this type of functional test, there are also certain advantages for a genetic screening approach. These include the objectivity of the results and the lack of requirement for viable cells. Assays measuring ATM function may also not identify all CLL tumours with *ATM* mutations. There may be tumours with a single *ATM* mutation and no loss of heterozygosity, where the mutation does not impact on the ATM function in the tumour cell. Furthermore, it is possible that an *ATM* mutation and / or a loss of an *ATM* allele may only be present within a sub-clone of the entire CLL tumour clone, and this could also lead to the appearance of retained ATM function using an assay that measures ATM activity on the whole tumour clone.

In this study the poor clinical outcome was related to the whole group of patients that had *ATM* mutations but, as discussed later, the identification of those tumours that have deficient ATM function might be the most clinically relevant with regard to treatment decisions.

7.1.7 Insight into the biology of CLL

The mechanisms through which prognostic markers might differentiate CLL tumours into subgroups have been studied for several existing biomarkers including *IGVH* status, CD38 and Zap70 and have thus increased the understanding of the biology of this tumour (Hamblin TJ *et al*, 1999, Damle RN *et al*, 1999, Dohner H *et al*, 2000, Wiestner A *et al*, 2003). In the second section of this discussion, I will consider the mechanisms by which *ATM* mutations affect the biology of CLL and how the status of the *ATM* gene might influence therapeutic decisions in the future.

7.2 The implications of *ATM* mutations in the biology of CLL tumours

ATM mutations may impact on the biology of B-CLL by having a role in the development of the CLL clone as well as by affecting the behaviour of the CLL clone once it has been established.

7.2.1 The role of ATM in the development of the CLL clone

The molecular basis underlying the genetic predisposition in CLL has not been established (Sellick GS *et al,* 2006). The *ATM* gene has been considered to be a candidate gene due to its high frequency in CLL tumours and also because AT patients are known to have a 200-fold increased incidence of lymphoid tumours, including those of B-cell origin. In addition, germ-line *ATM* mutations have been reported to occur in CLL tumours and certain specific *ATM* polymorphisms have also been found to associate with CLL patients versus controls (Stankovic T *et al*, 2002, Rudd MF *et al*, 2006). Furthermore, in addition to the potential role of germ-line *ATM* sequence changes in the predisposition to CLL, there is also a possibility that acquired *ATM* mutations could contribute to the development of the B-CLL tumour clone if they arose at or before the time of clonal transformation.

7.2.1.1 Germ-line *ATM* mutations

A previous study reported a higher than expected incidence of germ-line *ATM* mutations in CLL patients, based on the known incidence of heterozygous *ATM* mutation carriers in the UK. This raised the possibility that germ-line *ATM* mutations could predispose individuals to the development of CLL (Stankovic T *et al*, 2002). Speculation for a role of germ-line *ATM*

mutations in the causation of cancer is not new but a majority of research has focussed on the role of *ATM* mutations and sequence variants in the predisposition to breast cancer (Thortenson Y *et al*, 2003, Dork T *et al*, 2001). In this regard, a recent study has confirmed that individuals who inherit a single *ATM* allele containing a mutation which is known to cause ataxia telangiectasia when present in a homozygous or compound heterozygous state have a relative risk of for the development of breast cancer of 2.37 (95% confidence interval = 1.51-3.78, p=0.0003) (Renwick A *et al*, 2006)

To investigate the potential role of germ-line *ATM* mutations in CLL I made a direct comparison between the frequencies of germ-line *ATM* mutations in CLL patients with the frequency in a control population. I found that germ-line *ATM* mutations were present at a frequency of 2.5% in the CLL patients. This was lower than in the previous report where the prevalence of germ-line mutations was 6%, based on a population of 50 CLL patients (Stankovic T *et al*, 2002). In this study, I found germ-line *ATM* mutations were present at a frequency of 1.5% in the control population. These differences were not statistically significant and notably, the prevalence of germ-line *ATM* mutations in both the patient and control populations was higher than the previously estimated level of *ATM* heterozygote carriers in the UK population of 0.5% (Stankovic T *et al*, 1998). Clearly, due to the relatively small numbers of patients and controls analysed, these findings could have occurred by chance. In addition, due to the difficulty in distinguishing germ-line mutations from polymorphisms, the possibility remains that not all these sequence changes were in fact true mutations and highlights the importance of a

functional assay to prove that a *germ-line* sequence change has a detrimental effect on protein function.

An *in vitro* modelling system to assess the effects of specific sequence changes in the *ATM* gene has been developed in our laboratory and it would be important to use this system to assess the pathogenicity of the germ-line *ATM* mutations detected in this study. The modelling system involves the induction of expression of the ATM protein with the mis-sense change that is being investigated in an ATM null LCL cell line followed by the assessment of the activity of the corresponding ATM protein. The sequence change of interest is first generated using a site directed mutagenesis kit (Stratagene XL11). The mutant *ATM* coding sequence is then cloned into the multiple cloning site of the mammalian expression vector, pMEP4. This has an inducible promoter allowing the expression of the gene of interest to be controlled by, for example, the addition of the heavy metal ZnCl2. The construct containing the mutated *ATM* gene is stably transfected into an LCL which is null for ATM. ATM protein expression is then induced and the activity of the ATM protein can be determined by measuring the phosphorylation of downstream substrates following DNA damage with irradiation. This system has been successfully utilised to confirm the pathogenic nature of the 5228C/T sequence change, detected in this study, and the 7181C/T sequence change that I detected in a multiple myeloma tumour as part of an independent study (Barone G, personal communication).

The differences in the frequency of germ-line *ATM* mutations between the patient and control populations in this study were very small and suggest that if *ATM* germ-line mutations do predispose to the development of CLL it is

likely to be a low penetrance effect that would require a significantly larger case-control study to demonstrate. Such a low penetrance effect would be in keeping with the findings of several previous small studies that did not find clear evidence to support a role for germ-line *ATM* mutations in the predisposition for the development of CLL. These included a study of the relatives of AT patients, which did not identify any cases of CLL, and a study of CLL family pedigrees, which failed to observe any segregation of *ATM* mutations within affected family members (Thompson D *et al* 2005, Yuille M *et al*, 2002).

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7.2.1.2 *ATM* polymorphisms

A recently published study has provided evidence to support a potential role of *ATM* polymorphic changes in the causation of CLL (Rudd MF *et al*, 2006). This study used SNP arrays to perform a genome-wide comparison between polymorphisms in 992 CLL cases and 2707controls. The *ATM* gene was found to be one of only three genes that contained polymorphisms that were significantly associated with CLL patients. These two polymorphisms were the 2572T/C (F858L) change (OR=2.28, $P=3.1x10^{-5}$) and the 3161C/G (P1054R) change (OR=1.68, $P=6.0x10^{-4}$). Furthermore, the remaining two genes containing polymorphisms associated with CLL patients were also DNA damage response proteins, namely Chk2 and Brca1.

I compared the prevalence of polymorphisms in the *ATM* gene between CLL patients and controls in this study by using a screening approach that had the potential for detecting new or rare candidate polymorphisms that could have associations with CLL patients. Interestingly, although I found that

the 2572T/C alteration was more common in CLL patients than controls, the 3161C/G alteration was actually more frequent in the control population in this case-control series and neither polymorphism was significantly associated with patients or controls. The only sequence change that was significantly associated with CLL patients was the IVS62-8A/C polymorphism. Due to its intronic location the only possible potential mechanistic explanation for this finding is that this change could interfere with exon splicing. This has not been previously reported but warrants further investigation.

7.2.1.3 Acquired *ATM* mutations

Acquired *ATM* mutations could also contribute to the development of CLL if they occur at or before the time of clonal transformation. A previous study has reported on two patients that had *ATM* mutations detected in their tumour cells and which were shown to be absent from the patients' germ-line but present in non-tumour cells including T-lymphocytes and monocytes (Stankovic T *et al*, 2002). This suggested a potential role of acquired *ATM* mutations in the process of clonal transformation.

In this study I was able to confirm that a number of the acquired *ATM* mutations were already present in the tumour cells at diagnosis. The timing of the clinical diagnosis of CLL cannot be directly related to the initial transformation but these findings also suggest that these acquired *ATM* mutations could have contributed to the initial clonal transformation. Furthermore, in two patients there was more than one inactivating *ATM* mutation present in diagnostic material indicating that ATM function had already been lost in the tumour cells at this early stage. In one of these

patients CLL77 this was due to one germ-line mutation (1058delGT) and one acquired mutation (5224G/C (A1742P) and in the second patient CLL96, it was due to acquired mutations (2929Ins9, 5041A/G, 5044G/T) only.

7.2.1.4 Potential mechanisms by which *ATM* mutations contribute to the development of the B-CLL clone

The mechanism by which *ATM* sequence changes might contribute to the development of CLL is unknown.

It has been found that within the lymph node the level of ATM expression is much higher within the mantle zone compared to the germinal centres of the B cell follicles (Starczynski J et al, 2003). The level of p53 expression is also reduced within the germinal centre and this has been shown to be partly mediated via the transcriptional repressor BCL6 (Phan RT, Dalla-Favera R, 2004). It has been postulated that reduced p53 expression might be important in the germinal centre cells in order to allow cells to tolerate the DNA breaks required for immunoglobulin class switch recombination and somatic hypermutation without inducing a p53-dependent apoptotic response (Phan RT, Dalla-Favera R, 2004). In a similar manner, physiological down regulation of ATM might also occur to protect cells from DNA damage induced apoptosis in the germinal centre.

Interestingly, the B-cell tumour subtypes that have commonly been found to have *ATM* mutations, such as Mantle Cell lymphoma, are derived from B cells that usually have high ATM expression (Schaffner C *et al*, 2000, Starczynski J *et al*, 2003). By comparison, abnormalities of the *ATM* gene have not been found in follicular lymphoma and Hodgkin's lymphoma, which

are derived from B cells with low physiological ATM expression (Lossos IS *et al*, 2002, Lespinet V *et al*, 2005). This suggests that the inactivation of ATM through mutation has a greater tumorigenic potential within those B-lymphocytes that have high physiological ATM expression. However, conversely, both *ATM* and *TP53* mutations have been reported in diffuse large B cell lymphomas which are also derived from germinal centre lymphocytes and typically have low ATM and p53 expression (Gronbaek K *et al*, 2002, Leroy K *et al,* 2002). These lymphomas are also characterised by de-regulation of BCL6, which suggests that the kinetics controlling the regulation of p53 and the DNA damage induced apoptotic response might be important in the clonal evolution of this lymphoma subtype (Cattoretti G *et al*, 2005).

A previous study found that within CLL tumours, *ATM* mutations occurred exclusively in the subcategory that had unmutated *IGVH* genes. This led the authors to suggest that loss of ATM might be particularly important for the transformation at the pre-germinal stage of B-cell differentiation (Stankovic T *et al*, 2002). The findings in my study do not fully support this interpretation, since I found *ATM* mutations in CLL tumours with both unmutated and mutated *IGVH* genes although they were still significantly more common in CLL tumours with unmutated *IGVH* genes. The precise origin of the B-lymphocyte that becomes clonally transformed in CLL remains uncertain and microarray analysis has recently demonstrated that both *IGVH* mutated and unmutated CLL tumours have an expression profile that most closely resembles memory B cells (Rosenwald A *et al*, 2001, Klein U *et al*, 2001). CLL tumours with mutated *IGVH* genes are thus now believed to have been

derived from B-lymphocytes that are antigen experienced and have passed through the germinal centre, whereas CLL tumours with unmutated *IGVH* genes are thought to have arisen from B-lymphocytes in the marginal zone that have undergone a T-cell independent antigen-recognition process (Klein U, Dalla-Favera R, 2005). The ATM expression within these marginal zone lymphocytes is not known but one could speculate that if defects in ATM expression do indeed contribute to the clonal transformation process in *IGVH* unmutated CLL tumours there might be high physiological ATM expression in these B-lymphocytes.

It is interesting that, while *ATM* mutations are present at a high frequency in CLL tumours, B-CLL has not been reported within individuals with AT. One explanation for this finding is that AT individuals have a short life expectancy and therefore do not live for long enough in order to develop CLL (Taylor AM, and Byrd P, 2005). However, the rarer MCL and T-PLL, which are also characterised by high numbers of sporadic *ATM* mutations, are both seen at increased frequencies in AT patients. Furthermore, T-PLL is also seen in a younger age group in AT patients compared to sporadic cases (Taylor AMR et al, 1992). The principal mechanism thorough which loss of ATM activity is believed to predispose to the development of lymphoma in AT patients is through the development of chromosomal translocations consequent of the defective repair of DNA DSBs during VDJ recombination (Taylor AM *et al,* 1975). Since, the formation of the specific chromosomal translocations involving the T-cell receptor or the immunoglobulin genes, respectively, are believed to be key events leading to the formation of the T-PLL and MCL clones, it might be predicted that AT patients would be particularly

predisposed to the development of such lymphoid tumours (Taylor AM et al, 1992, Camacho E et al, 2002). By comparison, chromosomal translocations are relatively uncommon in CLL tumours and when present they are often late events occurring after the initial transformation process. Thus, an alternative explanation for the observation that CLL is not over represented in AT patients is because the development of chromosomal translocations is not vital for the initial clonal transformation process (Klein U, Dalla-Favera R, 2005, Finn WG *et al*, 1998, Mayr C *et al*, 2006).

From this discussion it can be seen that the extent to which *ATM* mutations actually predispose and contribute to the transforming event in CLL tumours is still uncertain. Nevertheless, the possibility remains that *ATM* mutations, either germ-line or acquired, could predispose to the transformation process through the failure to activate an apoptotic response in a B-lymphocyte that has acquired site non-specific DNA damage. As discussed below, it is possible that the loss of ATM function might occur as part of a multi-step process in the ontogeny of CLL, and that this step-wise loss of ATM function might be initiated both before and after the transformation of the B-cell clone.

7.2.2 The role of ATM in the biology of the established CLL clone

Whether or not *ATM* mutations have a role in the development of CLL, the marked effect of *ATM* mutations on clinical outcome in CLL patients that I detected in this project suggests that they certainly have an impact on the biology of the disease once the tumour clone has developed.

7.2.2.1 Mechanism of action of *ATM* mutations in CLL tumours

The mechanism of action by which *ATM* mutations might impact on the behaviour of the CLL clone has been previously investigated. As described earlier, CLL tumours with bi-allelic *ATM* mutations have been shown to fail to up regulate p53 or p21 following DNA damage with IR, and in addition they have also been shown to exhibit a defective apoptotic response to DNA damage in the form of DSBs (Pettitt AR *et al*, 2001). This apoptotic defect is less pronounced than the defect in apoptosis seen in CLL tumours with *TP53* mutations and I confirmed this previously observed pattern of apoptotic responses in this project (Stankovic T *et al*, 2002). Furthermore, the pattern of defective apoptosis in tumours with *ATM* mutations that is intermediate between tumours with *TP53* mutations and those with wild type *ATM* and *TP53* genes mirrors the pattern in clinical outcome that I have shown in this study according to the same genetic subgroups.

My results, and previously published studies, suggest that an important mechanism through which *ATM* mutations exert a pathogenic effect in CLL tumours is through impairment of apoptosis following DNA damage. However uncertainties have remained as to whether or not these functional effects relate only to tumours with abnormalities of both *ATM* alleles or whether there were also functional consequences of mono-allelic *ATM* defects in CLL tumours (Stankovic T *et al* 2002, Stankovic T *et al* 2004).

7.2.2.2 A deletion of an *ATM* allele in the absence of a mutation

I studied the early phosphorylation responses after DNA damage with irradiation to assess the function of the ATM protein. I demonstrated that the

chromosome 11q deleted tumours that had a remaining wild type *ATM* allele had retained ATM function and exhibited phosphorylation of downstream substrates after IR that was comparable to the responses in tumours with two wild type *ATM* alleles. Although there was a degree of variation, the tumours with an 11q deletion but a second wild type *ATM* allele could be clearly distinguished from those 11q-deleted tumours with a mutation in the remaining *ATM* allele, which all had very low level ATM activity. This indicated that 11q-deleted tumours form two functional subgroups in terms of their ATM activity. In representative tumours I also demonstrated that this differential ATM activity translated into differential downstream responses. Notably, the up regulation of p53 and induction of p21 was proficient in 11q-deleted tumours with a second wild type allele but impaired in all the tumours analysed with a second mutant *ATM* allele.

There was a degree of variation in the size of the subclone that carried the 11q deletion between different CLL tumours. The prognostic significance of variations in the size of the subclone that carries the chromosome deletion has not been reported. Interestingly, in my series a higher proportion of cells with an 11q deletion was seen within the whole tumour population when the clone had a mutation of the second *ATM* allele in comparison to clones that retained a wild type *ATM* allele. Indeed, it was uncommon to find a tumour with only a small subclone of cells carrying an 11q deletion if that clone carried an additional *ATM* mutation. This pattern did not occur in any of the tumours where viable cells were available for functional analysis of ATM activity. This suggests that the development of an *ATM* mutation in a tumour cell with an 11q deletion might lead to a more aggressive or faster growing

subclone of tumour cells. Notably, however, there were also several examples of tumours that had between 80 and 100% of tumour cells carrying the 11q deletion despite a remaining wild type *ATM* allele. These tumours all had proficient ATM activity thus confirming that the single wild type *ATM* allele was sufficient for normal ATM function in these CLL tumours, at least at the level of sensitivity of the assays used in this study. This implies that the expansion of the subclone with the chromosome 11q deletion in these tumours may have been independent of the ATM activity.

7.2.2.3 One mutant *ATM* allele and one wild type *ATM* allele

There were several tumours that I identified in this project that had one mutant *ATM* allele but retained a wild type second allele. Assuming that the presence of this wild type *ATM* allele is sufficient for normal ATM-dependent DNA damage induced phosphorylation responses, as the results above indicate, then it would be expected that these tumours would also have retained ATM function. However, this group included four tumours that had deficient ATM-dependent phosphorylation responses after DNA damage and in three of these cases I was able to demonstrate that they also had impaired up regulation of p53 and induction of p21 expression. Furthermore, I confirmed that in three of these tumours there had been no new development of an 11q deletion to account for this pattern of functional response.

I considered the possibility that the mutations could be exerting a dominant negative effect. Since ATM exists as a dimer in the cell, such a concept is feasible and a dominant negative effect has been previously reported for certain *ATM* mutations (Scott SP *et al*, 2002). Dominant negative

effects occur with mis-sense mutations that result in the production of a full-length stable protein containing an amino acid alteration and, therefore, such an effect could apply to CLLs 119 and 75 in this study that contained the mis-sense mutations 9022C/T (R3008C) and 1009C/A (R337S) respectively. To investigate this possibility would require an in vitro modelling system similar to that described earlier to test the function of specific mutations. However, in this situation the vector containing the mutant *ATM* gene would be co-transfected with a vector expressing an *ATM* wild type gene into an ATM null cell line. This would allow the assessment of the effect of the mutant protein on the function of the wild type ATM protein.

7.2.2.4 Mechanism for poor outcome in patients with mono-allelic *ATM* defects

Interestingly, in this study the whole group of patients with an 11q deletion had a poor outcome and similarly, the entire group of patients with *ATM* mutations had a poor clinical outcome compared to CLL patients with neither of these defects. However, as discussed, a considerable proportion of each group only had a defect in one *ATM* allele and furthermore, the majority of these individuals with a mono-allelic defect had retained ATM function in their cells. In the group of patients with 11q deletions, those patients that had an additional *ATM* mutation showed a trend towards a poorer survival but this was not significant and in addition there was no significant difference in treatment free survival between the two 11q- deleted subgroups. Due to low numbers it was not possible to make a similar comparison in clinical outcome between patients with both an *ATM* mutant allele and a second *ATM* allelic

defect with those that had an *ATM* mutant allele and a second wild type *ATM* allele. Furthermore, this comparison would have been conceptually more complex due to the fact that four patients with one mutant and one wild type *ATM* allele had deficient ATM function.

My results therefore suggest that CLL patients with a mono-allelic *ATM* defect also have a poor outlook. One possible explanation is that defects in the two *ATM* alleles may be acquired in a stepwise manner in some CLL tumours. Therefore, tumours with bi-allelic *ATM* abnormalities could represent a later stage in the disease course compared to those with a mono-allelic change. Indeed, in two patients in this study I showed that the *ATM* mutations had arisen in tumour cells during disease progression. Each of these cases also had an 11q deletion but, because the timing of the loss of chromosome 11q was not known for either tumour, it was not possible to be sure that the two genetic events had occurred sequentially. However, another tumour that had been previously identified to have two *ATM* mutations (2114insA, 4393insA) in a separate study could be shown to have acquired these defects in a step-wise sequential manner. In this case, one of the mutations (2114insA) was present in a sample collected from the time of diagnosis and the other mutation (4393insA) was only detected in a sample collected several years later (Austen B *et al*, 2005). The late development of chromosome 11q-deletions in CLL has also been previously described suggesting that the development of an *ATM* mutation or an 11q deletion might occur in either order (Cuneo A *et al*, 2002).

The step-wise development of *ATM* allelic defects could also partly account for the partial correlation that I observed between tumours with an

ATM mutation and tumours with an 11q deletion. This is unlikely to explain this finding completely since in certain tumours both *ATM* alleles will be inactivated by mutations. Furthermore, in this study over half of the patients with an 11q deletion had a second wild type *ATM* allele even though these included a number of long-lived patients in which the sample analysed had been collected many years out from diagnosis. This, therefore, suggests that a significant proportion of patients with one *ATM* defect in their tumour cells may never acquire a second *ATM* allelic defect.

Further indirect evidence to support the concept of the sequential development of *ATM* allelic defects is related to the three-fold higher proportion of 11q-deleted tumours that had *ATM* mutations compared to unselected CLL tumours in this study. This suggests that loss of one functional allele at the *ATM* locus predisposes to the subsequent development and clonal expansion of tumour cells with a second *ATM* abnormality. Clearly, any tumour cells that are already heterozygous at the *ATM* locus will lose ATM function if they acquire a mutation in the remaining *ATM* allele. Loss of ATM function would then be predicted to render the tumour cell resistant to DNA damage induced apoptosis, thus providing a cellular survival advantage. This could then lead to expansion of a subclone that has acquired bi-allelic *ATM* defects and would be consistent with the observation in this study that there is frequently a larger subclone carrying an 11q deletion in those tumours that have an *ATM* mutation in the second allele compared to those that have a second wild type *ATM* allele.

Interestingly, in all three tumours where it was possible to confirm the late development of an *ATM* mutation, the patient had received treatment with

DNA damaging chemotherapeutic drugs between the date of the initial sample that lacked the mutation and the second sample where the mutation was present. Therefore, in these tumours the use of DNA damaging drugs may have provided an additional selective pressure for the survival of a tumour cell that had developed a second allelic *ATM* defect.

In order for the initial mono-allelic *ATM* defect that has occurred via an 11q deletion or *ATM* mutation to be maintained in the tumour clone they must presumably also convey a survival advantage to the cell. Although *ATM* mutations may have occurred at the time of clonal transformation, deletions of chromosome 11q are commonly acquired after the initial clonal transformation, as indicated by the fact that in many CLL tumours they are not present in the total tumour cell population. Therefore for cells carrying this deletion to have expanded to form a subclone they must have induced a survival or proliferative advantage. If ATM function is indeed entirely normal in these cells this survival signal might be independent of ATM function. The region of deletion on chromosome 11q spans 2-3 Mb and contains a number of genes (Stilgenbauer S *et al*, 1996). At the current time, no mutations have been found in any of these alternative candidate genes but heterozygosity at certain genetic loci could also have a pathogenic effect in CLL tumours (Auer RL *et al*, 2005, Celeste A *et al,* 2003). Therefore the short survival in some patients with an 11q deletion who do not have an *ATM* mutation could be due to a defect that is independent of ATM. Alternatively, it is possible that the 11q deletion does have some subtle effect on ATM function which was not detectable by the assay used in this project, and it is this effect that leads to the maintenance of the 11q deletion within the CLL clone. Nevertheless, the

tumour would still be predicted to attain a further survival advantage and behave more aggressively following the subsequent development of the second *ATM* defect.

In this study there were two tumours that had one mutant *ATM* allele and also carried a mutation in the *TP53* gene. It is therefore conceivable that heterozygosity at the *ATM* locus could have predisposed to the development of a mutation in an alternative DNA damage response, namely the *TP53* gene. Unfortunately, it was not possible to determine the sequential timing of these genetic events for either tumour. Since abnormalities in p53 have a more marked effect on apoptosis than *ATM* mutations, one might consider the possibility that there would be no additional survival advantage to a cell that already had a *TP53* mutation by the subsequent development of the *ATM* mutation. Notably, however, ATM activates numerous p53-independent as well as p53-dependent cellular pathways. Furthermore, the BH3-only domain pro-apoptotic protein BID, which is member of the BCL2 family of proteins that act downstream of p53 in the intrinsic apoptotic pathway, has recently been found to be a direct target for ATM dependent phosphorylation (Kamer I *et al*, 2005). So there may indeed be a further survival advantage to a CLL cell with a *TP53* mutation by the later development of a defect in ATM function. This concept is also supported by murine studies that have demonstrated that ATM and p53 are not congruent but rather can cooperate in their tumour suppressive functions (Westphal CH *et al*, 2002).

7.2.3. Effects of *ATM* mutations on the response to Fludarabine

The defect in DNA damage-induced apoptosis that I have observed following irradiation in the tumours with *ATM* mutations is likely to contribute to the poor clinical outcome in patients. For example, these tumours may fail to activate this DNA damage response pathway following treatment with certain chemotherapeutic drugs which would therefore lead to chemo-resistance. However, evidence for a direct effect of *ATM* mutations on the response to DNA damaging chemotherapeutic drugs is lacking.

In order to investigate the effects of *ATM* mutations on the response to fludarabine, I initially studied the role of fludarabine in the induction of DNA DSBs and the activation of the DNA damage response pathway. The mechanism of action of the purine analogue fludarabine relies on its incorporation into DNA. In non-cycling CLL cells, where there is no DNA synthesis, this incorporation might occur during DNA repair processes such as mismatch repair or base excision repair following spontaneous or drug induced DNA damage (Pettitt AR, 2003). Once incorporated into DNA, fludarabine has been shown to signal the activation of p53-dependent apoptosis (Achanta G *et al*, 2001, Rosenwald A *et al*, 2004, Rao VA, Plunkett W, 2001). However, the role of ATM in the response to fludarabine in CLL cells has remained uncertain.

In this project, I have demonstrated that fludarabine leads to the induction DNA DSBs in CLL tumour cells and that these DSBs, as indicated by γH2AX foci, are present after 8 hours of culture but are more frequent after 24 hours of culture with fludarabine. I also showed that fludarabine led to the activation of the DNA damage response pathway that resulted in the phosphorylation of substrates that were known to be targets of the ATM protein. Furthermore,

tumours with bi-allelic *ATM* defects failed to phosphorylate these substrates indicating that the phosphorylation responses were dependent on functional ATM in an analogous way to the responses induced by irradiation. These results imply that the loss of ATM function could not be compensated for by the activity of related proteins such as ATR, at least in terms of the phosphorylation of these target substrates in the CLL tumours cells. There is known to be some redundancy between the function of ATM and ATR in the slower kinetic responses to DNA DSBs and the lack of compensation for loss of ATM in CLL tumours might be explained by the fact that ATR has been shown to be down regulated in non-cycling CLL tumour cells (Lin K *et al*, 2002). Under these circumstances functional ATM might be important for both rapid and slower responses to DNA DSBs.

In line with the irradiation experiments, I have also demonstrated in this project that tumours that had a retained wild-type *ATM* allele had responses to fludarabine that were equivalent to tumours with two wild type *ATM* alleles. Thus the group of tumours with an 11q-deletion showed an ATM-dependent response not only to irradiation but also to DNA damage induced by fludarabine.

These results therefore provide evidence indicating that a decrease in the response to the DNA damaging chemotherapeutic agent fludarabine is indeed one reason for the poorer outcome in CLL patients with absent ATM function. Furthermore, the use of these agents in tumours with impaired ATM function could actually be detrimental since the delayed or defective responses to the DNA damage could promote the generation of further genetic defects and hence an acceleration of tumour progression. In addition, as discussed

earlier, using these agents in tumours with loss of one *ATM* allele might also be detrimental as they could provide a selective pressure for the emergence of subclones that have acquired a defect in the second *ATM* allele.

7.3 Final conclusions and clinical implications

As a part of my PhD project, I have demonstrated that the detection of *ATM* mutations in CLL tumours has prognostic value. With a prevalence of 12%, *ATM* mutations represent the commonest single gene defect to be identified in CLL and, indeed, the frequency of CLL tumours that acquire *ATM* mutations prospectively may be even higher. *ATM* mutations lead to an aggressive clinical phenotype resulting in a significant reduction in both TFS and OS in CLL patients and they provide prognostic information that is independent of age, clinical stage, the mutation status of the *IGVH* genes and *TP53* mutations.

The timing of the *ATM* mutations and their relationship with CLL tumours with an 11q deletion is interesting and provides clues into their mechanism of action in the pathogenesis of the disease. In certain cases, they represent germ-line changes and in other tumours appear to be present very early in the disease course raising the possibility that they might contribute to the initial process of clonal transformation. By comparison, they can also occur during disease progression and this appears to be more common when they occur as the second defect in the *ATM* gene, leading to the loss of cellular ATM activity.

There is only a partial correlation between tumours with mutations in the *ATM* gene and those with loss of one *ATM* allele via an 11q deletion at any

point in time. Thus, the category of tumours with an 11q deletion forms two distinct subgroups based on the status of the remaining *ATM* gene and this has functional significance in terms of the responses to DNA damage. Interestingly, the presence of an 11q deletion also appears to lead to an increased predisposition for the subsequent development of an *ATM* mutation.

ATM is an important DNA damage response protein and when inactivated there is impairment in the activation of p53 and p53-dependent responses, including apoptosis. This has important implications for the use of DNA damaging drugs in the treatment of CLL tumours. As demonstrated in this study, chemotherapeutic agents such as Fludarabine activate the ATM dependent DNA damage responses and there is impairment in this response in tumours with bi-allelic *ATM* defects. These results indicate that chemo-resistance is likely to be an important mechanism accounting for the poor outcome in CLL patients with *ATM* mutations.

The identification of tumours with *ATM* mutations would therefore predict patients that will have a poor clinical outcome and are likely to require early treatment for their disease. However, it would also identify those tumours that might be resistant to standard treatments and, as such, would benefit from alternative therapeutic strategies that do not involve the ATM/p53 damage response pathway. Functional assays could be used to readily identify tumours with impairment of ATM function, which will principally be tumours with bi-allelic *ATM* defects. Such assays could, therefore, be used before each treatment episode, to guide the choice of therapeutic agents.

There may also be clinical value in the identification of CLL tumours with mono-allelic *ATM* defects, either through a mutation or an 11q deletion. These tumours would be predicted to be sensitive to DNA damaging drugs, but there is the possibility that the use of these drugs might provide a selective pressure for the emergence of sub-clones that have acquired bi-allelic *ATM* defects, which would in turn be associated with a more aggressive cellular phenotype.

Future Work

An interesting aspect of this work that I would like to develop is the study of the proposed subgroups of 11q-deleted tumours based on the mutation status of the remaining *ATM* allele. Specifically, within the research group we have already initiated the *ATM* mutation screening of a cohort of 100 tumours that all have an 11q deletion, which were collected from CLL patients entering the recent UK CLL 4 trial. This group of patients were all previously untreated and the samples to be genetically analysed were collected prior to the patients starting one of three therapeutic options, namely chlorambucil, fludarabine, or fludarabine and cyclophosphamide, according to the trial protocol. Therefore, it will be possible to relate treatment responses in these patients with the mutation status of their *ATM* genes prior to starting treatment. Since all these patients had an 11q deletion, any *ATM* mutation detected will be expected to result in the loss of ATM function in the tumour cells.

In addition, we will continue expanding the cohort of non-trial patients with an 11q deletion to assess whether it is possible to demonstrate any significant differences in survival between the two subgroups of patients in a larger, more powerful study.

Viable cells from both patient series will also be used to compare responses *in vitro* to the chemotherapeutic drugs used in the CLL4 trial. We will be interested to assess whether there are differential responses to chlorambucil and cyclophosphamide in addition to the differential responses that I observed to fludarabine between the two 11q-deleted subgroups. Furthermore, we propose to study not only the early ATM-dependent

phosphorylation responses but also the downstream effects on p53 dependent transcription, as assessed by gene expression profiling, and the induction of apoptosis following treatment with each of these agents.

Where possible, it will also be interesting to assess serial samples from more patients to assess whether a step wise loss of functional *ATM* allele is a common phenomenon in these patients and, importantly, whether this might relate to the prior use of DNA damaging therapeutics. Given the role of ATM in the repair of DNA breaks, as well as the activation of apoptosis, it would also be interesting to assess whether tumours that lose ATM function are predisposed to the development of further genomic instability such as chromosomal translocations. This might also provide an explanation, in addition to the resistance to chemotherapeutics, to account for the significantly poorer outcome in patients with *ATM* mutations. Such potential genomic instability could be assessed via karyotyping or by using more sensitive techniques such as comparative genomic hybridisation or SNP arrays.

In this project, I also identified a group of tumours with an impairment of apoptosis following DNA damage with irradiation despite the presence of wild type *ATM* and *TP53* genes. The mechanism to account for this observation warrants further investigation. One explanation could be that there is up-regulated DNA repair in these tumours, as highlighted in a previous report, and it will be interesting to study repair assays, particularly the status of NHEJ and DNA-PK activity in these tumours (Deriano L *et al*, 2005). Alternatively there may be defects in the activation of p53-mediated downstream functions, based on differential interaction of p53 with specific promoters (Meek D,

2004). These possibilities will be investigated in our research group in the future.

One of the aims of this project was to investigate the contribution of germ-line *ATM* defects to the development of CLL. Due to limitations of the study, it was difficult to make firm conclusions with respect to this part of my project. Nevertheless, there remains the possibility that *ATM* mutations may have a low penetrance effect in the causation of CLL. The ongoing screening of CLL patients for *ATM* mutations in our laboratory will provide further cumulative information and any potential differences between the proportions of germ-line *ATM* mutations between CLL patients and controls can be re-assessed. As alluded to earlier, these alterations could also be modelled to confirm their pathogenic nature.

To aid *ATM* mutation analysis, we also intend to develop a FACS test to screen CLL tumours to allow the rapid detection of tumours with impairment in the DNA damage response. Those tumours with deficient responses will subsequently be screened for *ATM* mutations. This will allow the ongoing detection of tumour with mutations, which is important for our future research, but will also identify patients that may benefit from novel therapeutic agents, which bypass the ATM/p53 pathway, and are available as part of Phase 1 and 2 clinical trials.

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PE Applied Biosystems

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Pharmacia Ltd

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R&D systems Ltd

19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB

Roche Diagnostics

Roche Molecular Biochemicals, Sandhofer Strasse 116, D-68305 Mannheim, Germany

Santa Cruz Biotechnology

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Schering Health Care Ltd

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Sigma Aldrich Ltd

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Transgenomic Ltd

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Upstate Biotechnology

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