The *ATM* and *TP53* genes and their association with cancer

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To Eileen, my wife and friend, and to the rest of my family, thank you for all your support and encouragement.

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ABBREVIATIONS

Human gene symbols are given in accordance with The Genome Database nomenclature. Human genes are written in uppercase italic, while proteins are presented by the same letters in plain uppercase.

53BP1	tumor protein p53 binding protein, 1
A	Adenine
AFP	α-fetoprotein
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated gene
ATR	Ataxia telangiectasia- and rad 3-related
ATX	autotaxin
ARF	ADP-Ribosylation Factor related
BAI1	brain-specific angiogenesis inhibitor 1
Bax	BCL2-associated X protein
bp	Base pairs
BER	Base excision repair gene
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
C	Cytosine
CDK1	Cyclin-dependent kinase 1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (Alternatively; P21/WAF1)
cDNA	Complementary deoxyribonucleic acid
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
C-terminal	carboxy terminal
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DSB	Double strand break
DSBR1	Double strand break gene 1
ERBB2	erythroblastic leukemia viral oncogene homolog 2
FANCD2	Fanconi's anemia gene, complementation group D2
Fas	TNF receptor superfamily, member 6
FRAP	FKBP-rapamycin associated protein
FRDA	Friedreich ataxia gene
G	Guanine
GADD45	growth arrest and DNA-damage-inducible
GD-AIF	GD-apoptosis inducing factor
H2AX	H2A histone family, member X
HR	Homologous recombination
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
kb	kilo base
Killer/DR5	tumor necrosis factor receptor superfamily, member 10b
KU70	KU70 protein
Ligase IV	ATP dependent DNA ligase 4
LOH	loss of heterozygosity
Maspin	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5
MDC1	mediator of DNA damage checkpoint 1
MDM2	mouse double minute 2 homolog isoform
MMR	Mismatch repair gene
MRE11	Meiotic recombination 11
MYC	myelocytomatosis oncogene
mRNA	Messenger ribonucleic acid
NBS1	Nijmegen breakage syndrome gene 1
NER	Nucleotide excision repair gene
NHEJ	Non-homologous end-joining
Noxa	phorbol-12-myristate-13-acetate-induced protein 1
N-terminal	amino-terminal
$p14^{ARF}$	cyclin-dependent kinase inhibitor 2A
p21	see CDKN1A
P53AIP1	p53-regulated apoptosis-inducing protein 1
PCR	polymerase chain reaction
Perp	PERP, TP53 apoptosis effector
PI-3K	Phosphatidylinositol-3-OH-kinase
PIDD	leucine-rich repeats and death domain containing
Puma	BCL2 binding component 3
PTT	Protein truncation test
RAD N	Radiation protein number N
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
SMC1	Structural maintenance of chromosomes gene 1-like 1
SIR	Standardized incidence ration
T	Thymine
TNF	tumor necrosis factor
TGFBR2	transforming growth factor, beta receptor II
TP53	Tumor protein 53 gene
TRRAP	transformation/transcription domain-associated protein
TSP1	thrombospondin 1
WAF1	see CDKN1A

Full and alternative names of genes can be found at the Online Mendelian Inheritance in Man web site at John Hopkins University
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

LIST OF ORIGINAL PAPERS

I. **Bregård, A,** Geitvik, GA, Jansen, L, Berg, M, Vu, P, Rødningen, O, Stray-Pedersen, A, Olsen, JH, Kleinerman, R, Robsahm, TE, Tretli, S, Nesland, J, Børresen-Dale, A-L *Pedigree Analyses, ATM Mutations and Cancer Risk in Norwegian Families with Ataxia Telangiectasia. Manuscript (Revised version will be submitted)*

II. Olsen, JH, Hahnemann, JMD, Børresen-Dale, A-L, Tretli, S, Kleinerman, R, Sankila, R, Hammarsström, L, Robsahm, TE, Kääriäinen, H, **Bregård, A**, Brøndum-Nielsen, K, Yuen, J, Tucker, M. *Breast and other cancers in 1445 blood relatives of 75 Nordic patients with ataxia telangiectasia. Submitted.*

III. Langerød, A, Bukholm, IRK, **Bregård, A**, Lønning, PE, Andersen, TI, Rognum, TO, Meling, GI, Lothe, RA, Børresen-Dale, A-L. *The TP53 Codon 72 Polymorphism May Affect the Function of TP53 Mutations in Breast Carcinomas but not in Colorectal Carcinomas* Cancer Epidemiology, Biomarkers and Prevention 11:1684-1688, 2002

PREFACE

Sir Francis Crick said: “DNA is, in fact, so precious and so fragile that we now know that the cell has evolved a whole variety of repair mechanisms to protect its DNA from assaults by radiation, chemicals and other hazards. This is exactly the sort of thing that the process of evolution by natural selection would lead us to expect” (1988, What Mad Pursuit. Basic Books: New York). As Crick pointed out, damage to DNA sparks a highly regulated and strictly controlled process in a normal cell. If some of these processes are interrupted, it may result in neoplasm and tumour growth.

The main objective of this work has been to investigate genetic alterations in two genes encoding proteins involved in cell cycle control and the DNA damage response machinery. The *ATM*[a] (Ataxia Telangiectasia Mutated) gene is analyzed for mutations in AT (Ataxia Telangiectasia) patients and their relatives to estimate the cancer risk for *ATM* heterozygous individuals. These data are also included in a large Nordic epidemiologic study. The *TP53* gene is analyzed for a polymorphism in patients with colon and breast cancer to assess whether this polymorphism is associated with mutated *TP53* in the cancers.

[a] The name of the gene can be confusing. The *ATM* is mutated in AT patients. However, *ATM* is the name of the functional wild type gene.

GENETICS AND CANCER – AN OVERVIEW

Cancer is a genetic disease – a result of complex interactions between genetic and non-genetic factors like environmental influence. This disease group is a consequence of accumulation of many different genetic changes, like alterations in genes involved in cell proliferation, regulation, and defect DNA repair systems. Predisposed individuals carry genetic alterations, including mutations, polymorphic variants and epigenetic changes like methylation. These germline alterations can initiate cancer development with high or low penetrance. Furthermore, additional somatic genetic changes are required for malignant tumour development.

Three groups of genes are associated with cancer development when they are altered: proto-oncogenes, tumour suppressor genes, and DNA repair genes.

Proto-oncogenes stimulate appropriate cell growth and cell division under normal conditions, as required for the continued turnover and replenishment of the skin, gastrointestinal tract and blood, for example. Mutations in these proto-oncogenes (also known as oncogenes) act dominantly and can lead to a gain in function. Cells with activated oncogenes continue to grow (or refuse to die) even in the presence of growth inhibition signals. *ERBB2*, *MYC* and *HRAS* are examples of oncogenes associated with breast cancer.

While oncogenes encode proteins that stimulate cell division, the tumour suppressor genes encode proteins with restraining effects. The main role of tumour-suppressor genes is to depress cell proliferation, by either inhibiting cell growth through the cell cycle or by promoting programmed cell death (also known as apoptosis). When several of these growth brakes are rendered non-functional through epigenetic and genetic changes and oncogenes are activated, the cell becomes malignant. Examples of tumours suppressors are the genes encoding the retinoblastoma protein, inactivated in retinoblastomas and *TP53*, which inhibits cyclin-dependent kinases and is inactivated in many different tumours. An epidemiological study performed on retinoblastomas in 1971 led to the hypothesis that development of the disease required two successive mutations (Knudson, 1971). The hypothesis has been generalised (Knudson Jr., 1978) and it is referred to as the “two hit model” for tumourigenesis.

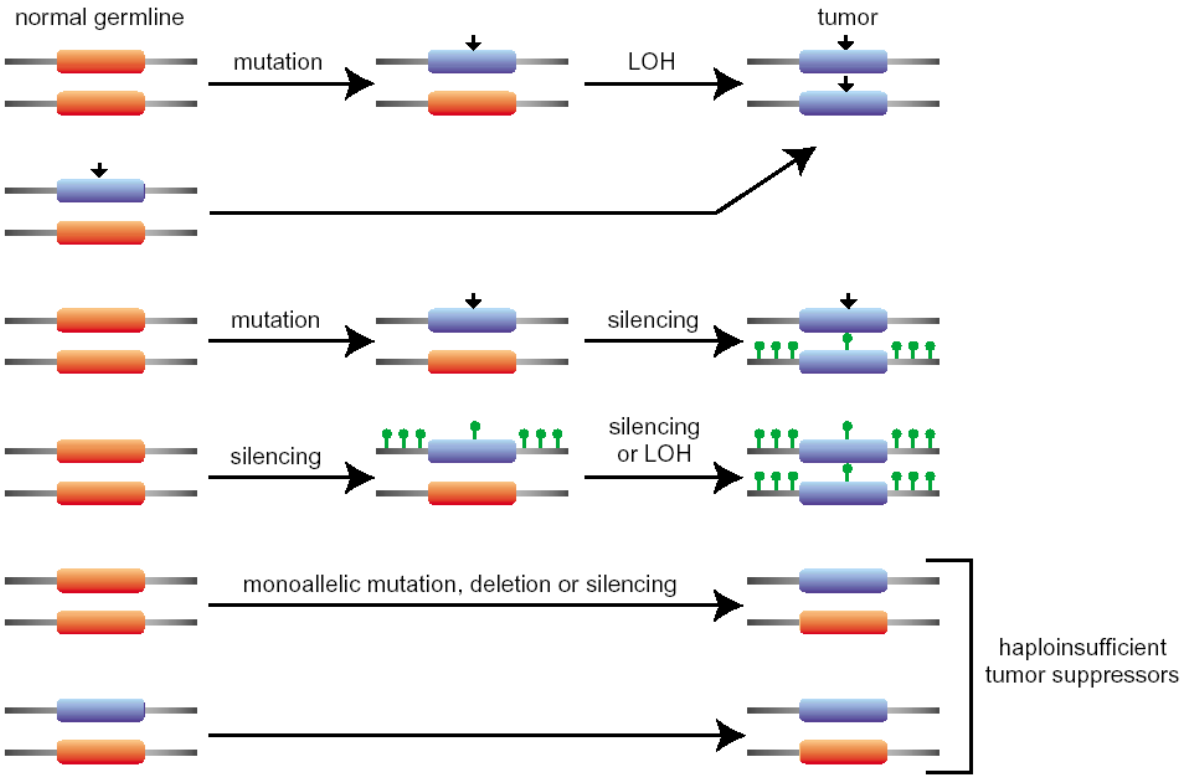

Figure 1: Knudson's two-hit hypothesis leading to inactivation of tumour suppressor genes – **B**lue bars indicate inactivated alleles – they can be germline or somatic; LOH is loss of heterozygosity by non-disjunction, mitotic recombination or deletion; green dots indicate gene silencing by promoter methylation. Some tumour suppressors genes do not need two hits to infer an increased cancer risk. (Balmain *et al*., 2003)

The first step in the tumour suppressor gene inactivation is a mutation (alternatively loss or methylation) of one of the two alleles. This mutation is transmitted in the germ-line patients with the familial type of the disease, whereas it occurs somatically in sporadic cases. The second mutation or "hit" occurs somatically in both hereditary and sporadic cases. For a tumour suppressor gene to loose its inhibitory effect on cell proliferation, both parental gene copies have to be inactivated or lost.

Unlike oncogenes and tumour-suppressor genes, repair genes do not control cell birth or death directly. They repair various DNA damage and therefore indirectly control the rate of mutation in the genome. These genes work to keep genetic alterations to a minimum, and when they are inactivated, the probability that cells acquire mutations in oncogenes and tumour-suppressor genes is increased, thus increasing the risk for development of benign and malignant tumours. Examples are nucleotide-excision repair genes (*NER*), mismatch-repair genes (*MMR*), and base-excision repair genes (*BER*) whose inactivation can lead to

susceptibility to skin and colon tumours, respectively. (Vogelstein *et al*., 2000) These genes are responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens. As with tumour suppressors, both alleles of stability genes generally must be inactivated for a physiological effect to result (Vogelstein and Kinzler, 2004)

While Knudson's hypothesis was developed before the era of molecular biology, Evidence from the recent molecular era also indicates that cancers can arise from small numbers of events that affect common cell birth and death processes. Retinoblastomas are still believed to follow the two hit hypothesis. Colon cancer can also arise from few genetic changes as shown in figure2.

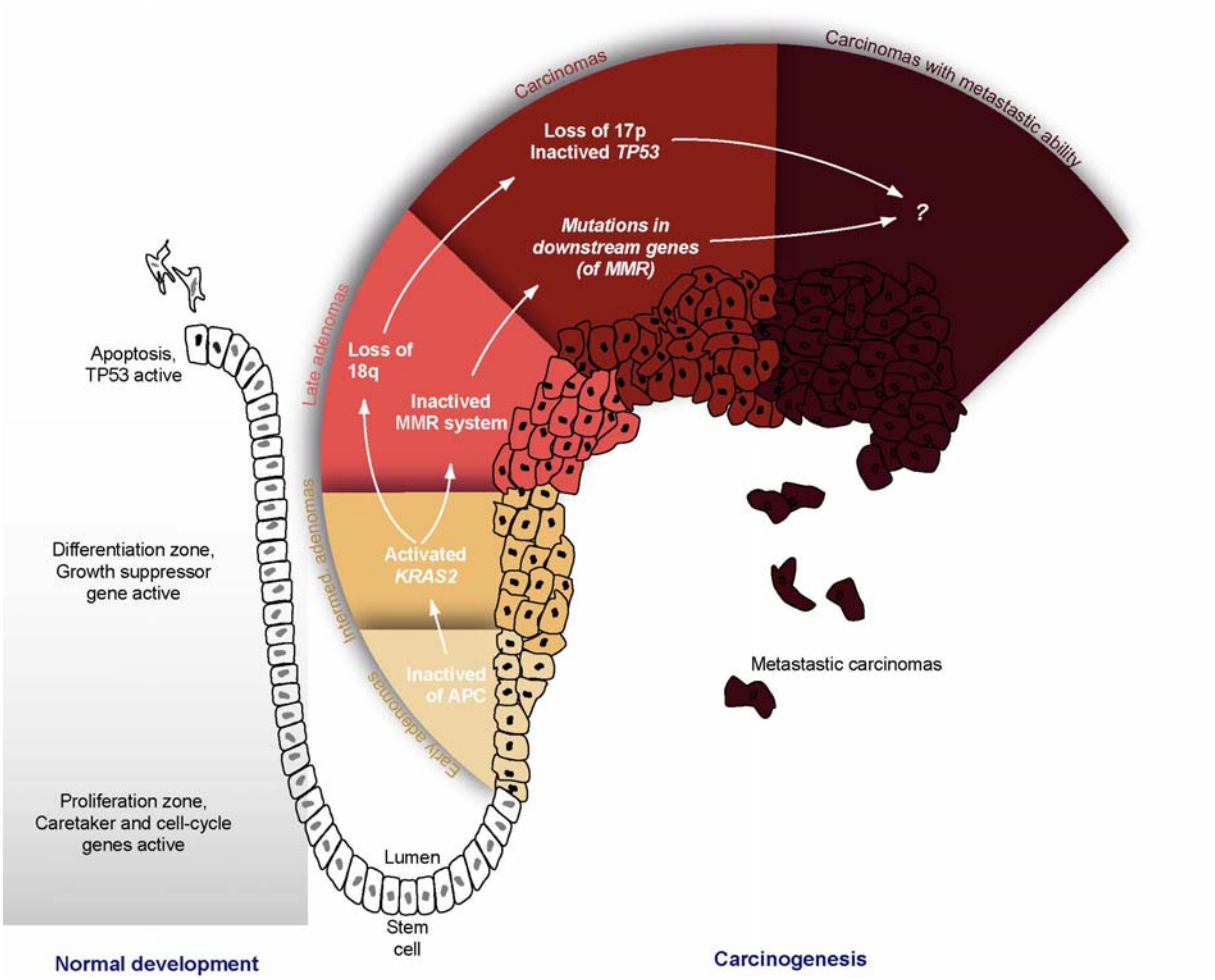

Figure 2: The development of colorectal cancer – has two distinct phenotypes: MSI and CIN. MSI is characterized by defects in the mismatch repair systems, leading to mutation in downstream genes such as *TGFBR2*. CIN is characterized by several chromosome aberrations including deletions of chromosome arms 17p and 18q, and mutation in *TP53*. MSI: microsatellite instability; CIN: chromosome instability; MMR: mismatch repair genes (Diep, 2002).

Any change in base sequence in DNA is considered a mutation. However, common variants (> 1% of the population) are determined polymorphisms. Mutations and polymorphisms may be harmful, helpful or neither to the cell(s) that have them. Mutations or polymorphisms that occur in a germline cell can be passed on to offspring, whereas changes in a somatic cell to the next generation of cells. Mutations and polymorphisms can be located in coding regions of DNA (exons) or the non-coding regions (introns - include splice sites). Therefore, alterations in DNA sequence can have a variety of consequences for protein expression and function, including loss or gain of function.

Damage to DNA

Crucial to the maintenance of a cell's genomic integrity is the cellular response to DNA damage. The cell cycle is very accurately controlled in a normal cell to avoid accumulation of genetic changes. Ideally, an identical copy of the genome is passed onto the next generation of cells. There are two basic responses that can be activated due to DNA damage: DNA repair systems and apoptosis. If the amount of damage overwhelms the capacity of the survival response machinery, apoptosis is initiated (Figure 3).

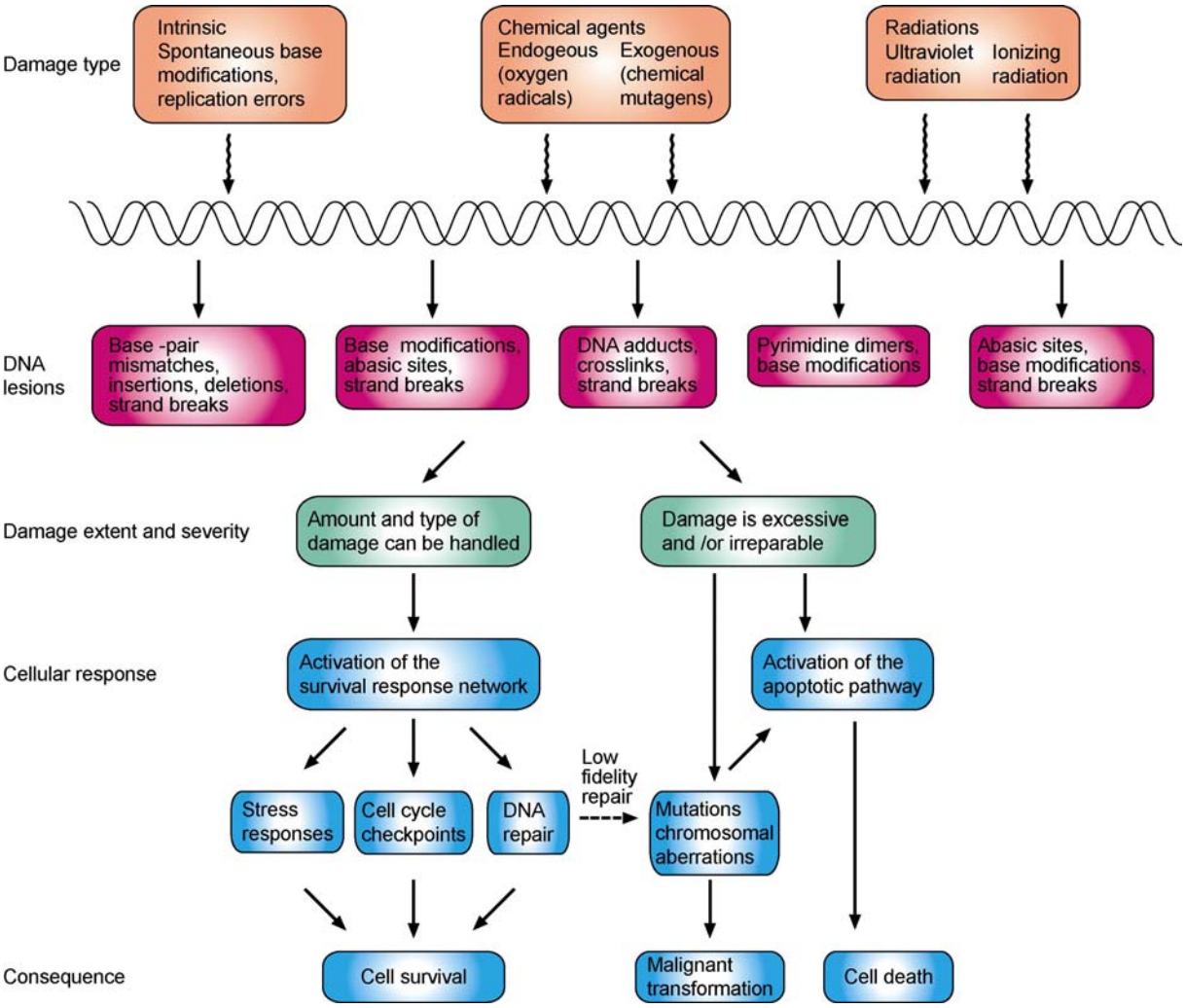

Figure 3: Cellular response to DNA damage. Different types of DNA damage are handled differently according to the type of lesion. It is not completely understood how the cell decides which of the two mechanisms to use. (Modified from Shiloh, 2003).

However, if the cell cycle control- and/or the DNA repair-mechanisms fail to respond appropriately, the cell may be allowed to pass into mitosis with faulty DNA. This

compromises tissue and organ function and can lead to uncontrolled cell division, tumour growth, and other degenerative diseases (Abraham, 2003).

Damage to DNA by ionizing radiation

One of the greatest external threats to genomic integrity and cellular viability is ionizing radiation (IR). IR is an important tool in medical diagnostics and treatment. Normal x-rays such as mammography or enhanced versions such as computer tomography are diagnostic tools based on the use of ionizing radiation. Furthermore, IR is often used in various cancer treatments alone or in combination with surgery and chemotherapy. IR includes alpha and beta particles, gamma- and x-rays and consists of high-speed electrons that have sufficient energy to eject electrons (excitation) from atoms in a cell. Ionization and excitation can lead to breakage of chemical bonds, including DNA, and the formation of free radicals (Steel, 2002). The most severe consequence of IR exposure to the cell is single- and double-strand DNA breaks (DSBs). Additionally, the risk of double strand DNA breaks is also elevated by other factors like ultra violet (UV) light, chemical mutagens, and reactive oxygen intermediates.

ATM

Ataxia Telangiectasia

Ataxia Telangectiasia is a rare autosomal recessive disease, caused by mutations in the *ATM* gene. The disease incidence is in the range of 1:40 000 – 1:100 000 live births depending on ethnic groups (Swift et al, 1986). In Norway, twenty-one AT cases have been reported (Olsen, submitted). In the American population, 1% (2,5 million people) is estimated to be carriers of a defect *ATM* gene[b].

Typical clinical symptoms are telangiectasias on the eyes (dilated blood vessels), cerebellar degeneration, hence ataxia, extreme IR sensitivity, immune defects, chromosomal complexity, and predisposition to various cancers, especially leukaemias (Gatti, 1991). *ATM* can be mutated at several locations within the gene leading to different degrees of the disease, some more severe than others. Rare cases of AT patients with milder manifestations of the clinical or cellular characteristics of the disease have been reported and have been designated as "AT variants". These cases of AT comprise a heterogeneous group characterized by later onset of clinical symptoms, slower progression, extended life span compared to most AT patients, decreased levels of chromosomal complexity, and cellular radio-sensitivity (Saviozzi *et al*., 2002). In these patients, telangiectasia and/or immunodeficiency may be absent, while the neurologic features are present.

AT is often discovered when the patient is between 1 and 3 years of age. The first signs of the disease are difficulties with control of body posture and body movement. Telangiectasias (tiny, red "spider" veins), which appear in the corners of the eyes or on the surface of the ears and cheeks, are characteristic of the disease, but are not always present and generally do not appear in the first years of life[c]. However, by the age of five, these dilated blood vessels in the white of the eye normally occur.

It was early noted that children with ataxia telangiectasia were radiation sensitive when two AT children died of acute radiation sickness after receiving conventional doses of

[b] Ataxia Telangiectasia Society, http://www.atsociety.org.uk, Accessed 15. December 2004.
[c] National institute of neurological disorders and stroke http://www.ninds.nih.gov/disorders/a_t/a-t.htm#What_is Accessed 5. January 2005

radiotherapy for their lymphomas (Cunliffe *et al*., 1975). More recently, testing of radio-sensitivity on cells from patients has been proposed as an effective way to diagnose AT early, as cells from AT patients are more susceptible for radiation than cells from healthy persons (Sun *et al*., 2002).

Approximately half of the AT patients have immune problems, resulting in repeated colds and pneumonias. The immunodeficiency is caused by decreased levels of immunoglobulin A, G and E (IgA, IgG, and IgE) (Stray-Pedersen *et al*., 2000). Levels of serum alpha-fetoprotein (AFP) are elevated in 95% of patients, making this an important additional diagnostic method for AT patients. Why AFP levels are elevated, remains unclear. It has been proposed that AFP has a suppressor effect on the development and function of the immune system.

Other features of the disease may include mild diabetes mellitus, premature greying of the hair, difficulty swallowing, and delayed physical, and sexual development (Ristow, 2004; McKinnon, 2004). However, children with AT usually have normal or above normal intelligence.

There is no curative treatment for AT and, currently, no way to slow the progression of the disease. Therefore all treatments are symptomatic and supportive. Physical and occupational therapy may help maintain flexibility. Speech therapy may also be needed. Gamma-globulin injections may be given to help supplement a weakened immune system. However, only a few patients reach the age of 50. The cause of death in AT is often pneumonia or chronic lung disease, which might result from immunodeficiency and defects in chewing and swallowing owing to progressive neurological impairment (McKinnon, 2004).

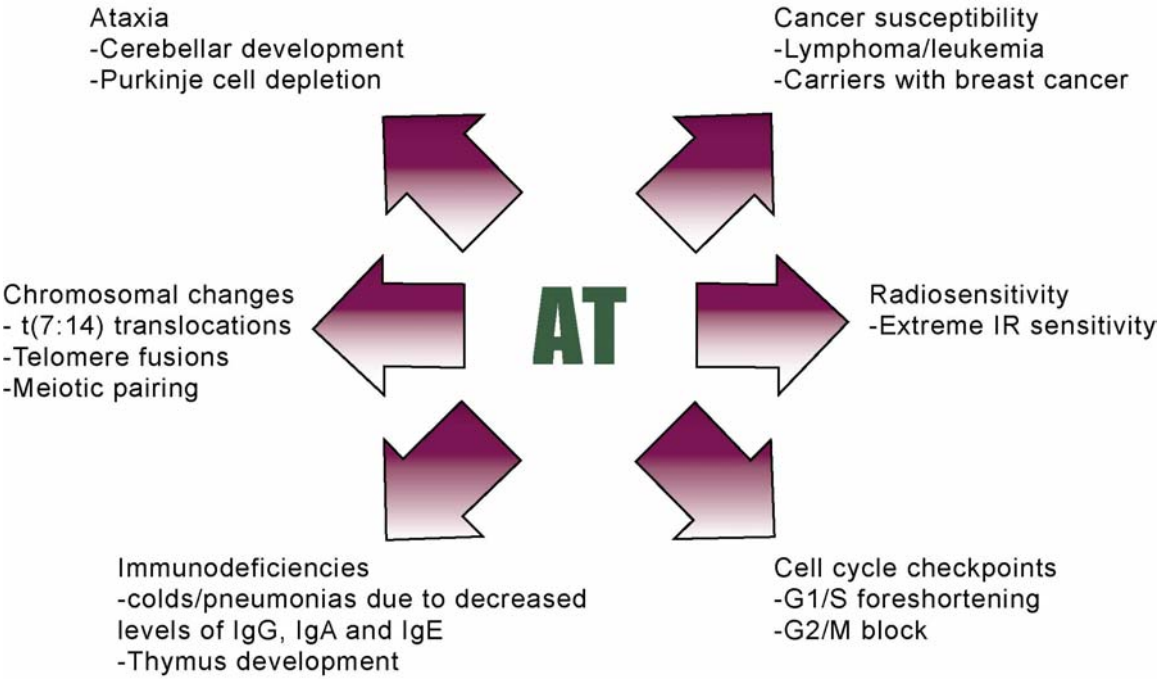

Figure 4: Various cellular features and phenotypes of AT patients[d]

AT patients and cancer

AT patients have approximately 100 times higher risk of developing cancers than unaffected age matched subjects (Swift *et al*., 1991). Cancer is a disease that results from alterations in the genome, therefore many individuals who suffer from syndromes that are characterized by defects in DNA damage responses, are also cancer prone (Hoeijmakers, 2001; van Gent *et al*, 2001). About 38% of the young AT individuals develop cancer. However, despite the nervous system being markedly affected in AT, the tumour types occurring in this disease are primarily lymphoma or leukaemia (Gumy-Pause *et al*, 2004). Typical cytogenetic changes seen in tumours from AT individuals often involve aberrant oncogenic rearrangements at the T-cell receptor loci. The occurrence of these tumours underscores the requirement for ATM to ensure high-fidelity immunoglobulin-gene recombination after the normal DNA breakage and processing that occurs during immune system maturation (Liao & Van Dyke, 1999; Perkins *et al*, 2002). Generally, lymphomas in AT patients tend to be of B-cell origin (B-CLL), whereas the leukaemias tend to be of the T-CLL type. Other solid tumours, including medulloblastomas and gliomas, occur with increased frequency in AT (Gatti *et al*., 1991).

[d] ATM mutation database http://www.vmresearch.org/bri_investigators/atminfo.htm#cycle Accessed 31. November 2004

The *ATM* and its gene product

ATM, localised to chromosome band 11q23.1 (Gatti *et al*., 1981), is 150kb long and consists of 66 exons (Uziel *et al*., 1997; Platzer *et al*., 1996), resulting in approximately 12 kb of mRNA (McKinnon, 2004) encoding a large protein consisting of 3056 amino acids and with an estimated molecular mass of 350 kDa (Savitsky *et al*., 1995a). This protein is a member of a large conserved eukaryotic family of protein kinases involved in functions like cell cycle control, DNA repair, and recombination (Savitsky et al. 1995b). In mammals this family includes DNA-damage response proteins such as ATM, ATR, ATX/SMG-1, DNA-PKcs, TRRAP and FRAP. Most of the proteins in this family, including ATM, possess a serine/threonine kinase activity (Shiloh, 2003). All proteins in this family contain a domain with motifs that are typical for the lipid kinase phosphatidylinositol 3-kinase (PI-3K) (Savitsky *et al*., 1995b). They are therefore named PI3K-like protein kinases. (PIKKs).

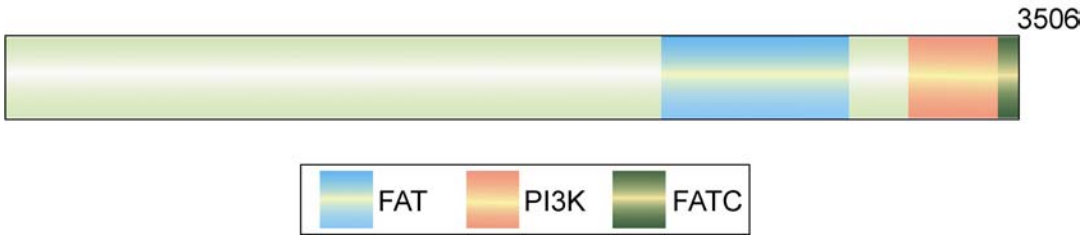

Figure 5: Functional domains in the ATM protein (Modified from Shiloh, 2003)

FAT, PI3K, and FATC are common motifs in the human members of the PIKK family (Fig. 5). The PI3K domain of the ATM protein harbours the catalytic site of the active kinase. The *ATM* gene product resides mainly in the nucleus of dividing cells. In undamaged cells the protein exists in an inactive form as a dimer. In this state the FAT domain of the other monomer blocks the catalytic site (Figure 6). As a consequence of rearrangements in the chromatin structure (for example caused by DSBs), the PI3K domain autophosphorylates the FAT domain, which in turn releases the two molecules from each other's grip. This leaves two fully activated ATM monomers.

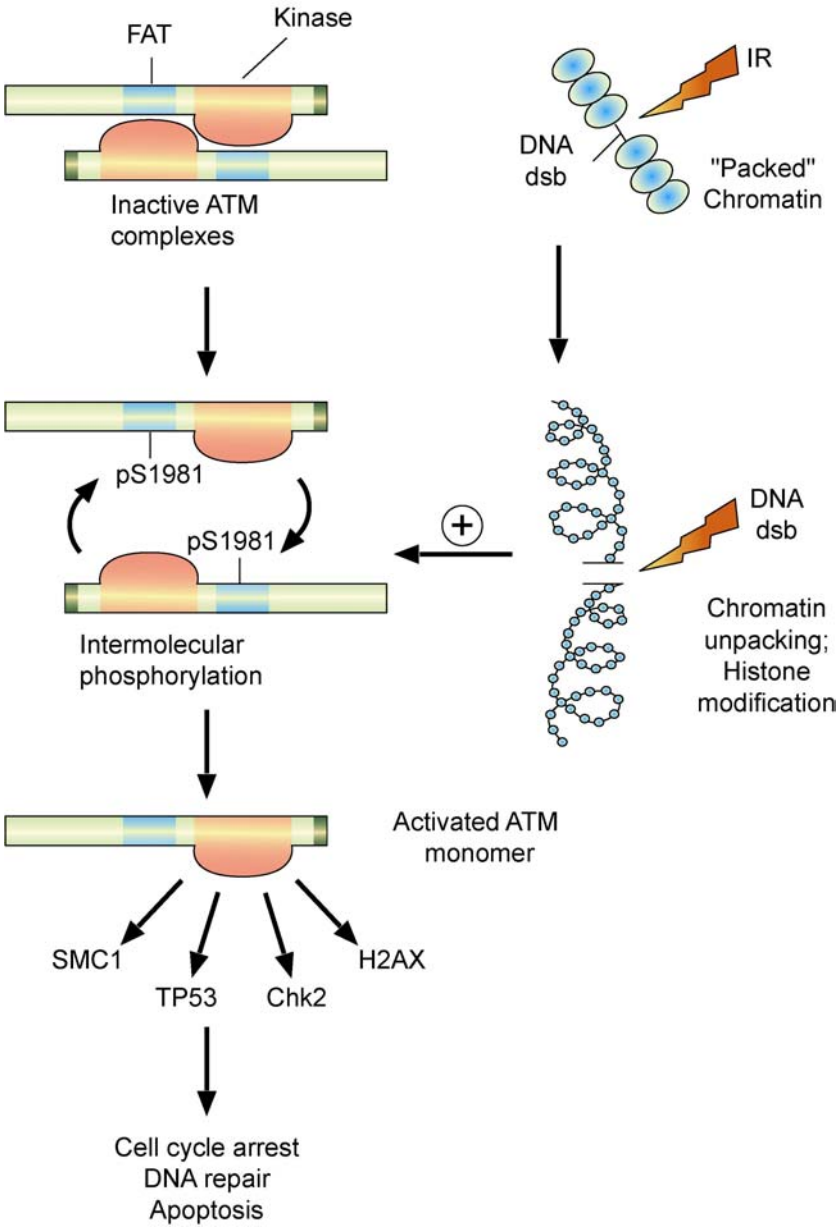

Figure 6: ATM activation As a dimer, the FAT domain in one of the monomers blocks the kinase-containing PI3K domain of the other monomer. As a response to changes in the chromatin structure, each ATM molecule phosphorylates the other on a serine residue at position 1981 within the FAT domain. This releases the two molecules from each other's grip and the monomers become highly active. ATM becomes active a few minutes after the occurrence of DNA damage (Modified from Abraham, 2003).

ATM – at the apex of DSB signaling

A large number of responses are initiated by double strand breaks (Figure 7). The ATM protein is essential in this response to DNA DSBs. The phenotype caused by loss-of -function mutations in *ATM* indicates that ATM is at the apex in the DSB response cascade (Shiloh, 2003).

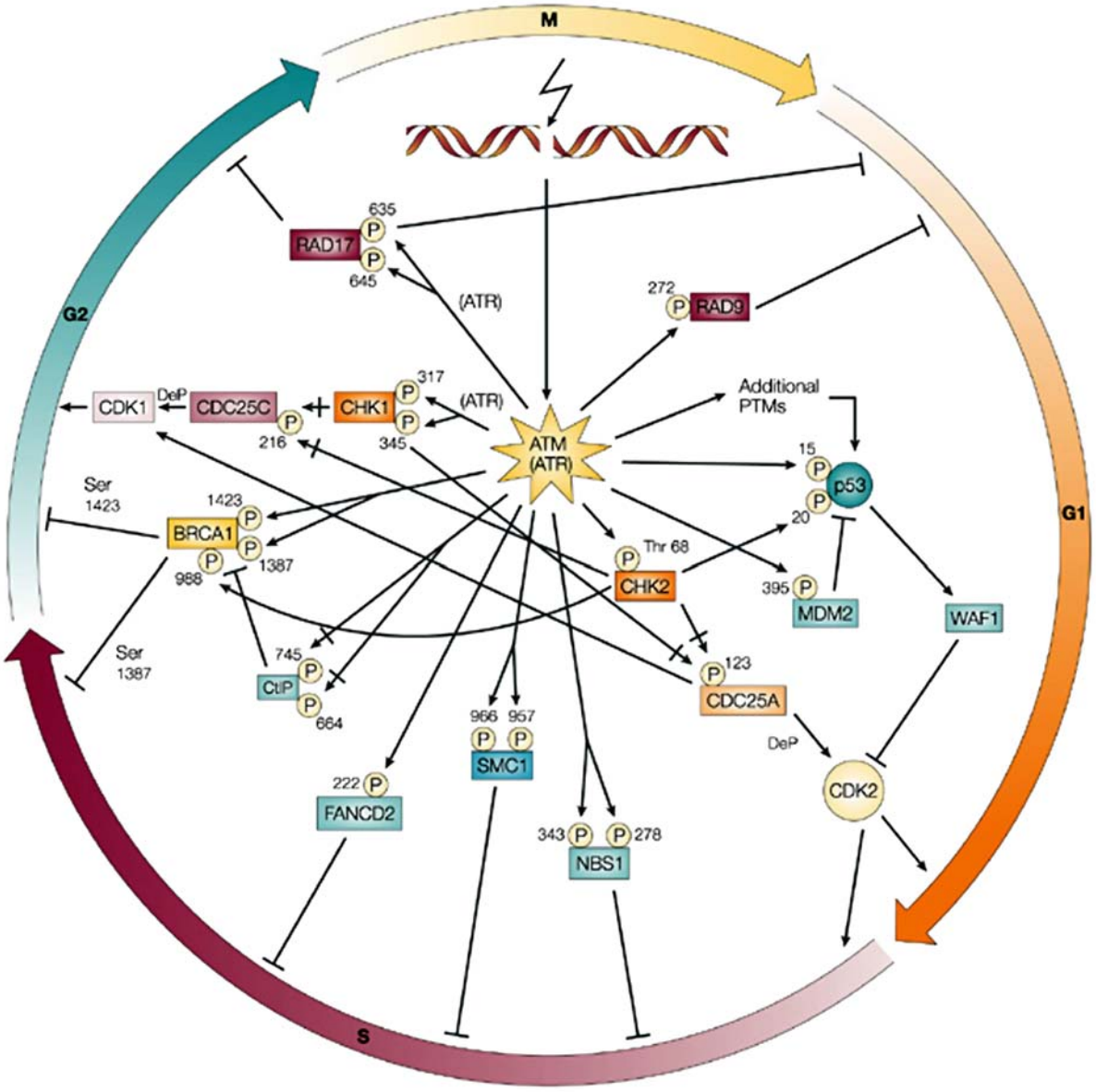

Figure 7: ATM-mediated activation of cell-cycle checkpoints in response to DSBs. Arrows indicate phosphorylation/stimulation. Arrows with a line through indicates inhibitory phosphorylations. T-shaped lines indicate cell cycle arrest. ATM regulates most of these pathways except from the CHK1 and the RAD17 pathways, which is regulated by ATR. It is also believed that ATR becomes more important at later stages to maintain all of these pathways (Shiloh, 2003).

Repair of DNA double strand breaks

ATM becomes highly active only a few minutes after DSBs. In response to a DSB, ATM divides into two fractions: one chromatin bound and one free portion that are recruited to the DSB site. The free ATM strongly adheres to the DSB site and it is believed that it serves as a platform for enzymatic reactions that takes place at those sites (Andegeko *et al*., 2001). ATM phosphorylates numerous substrates, either enhancing or repressing their activities (Figure 7).

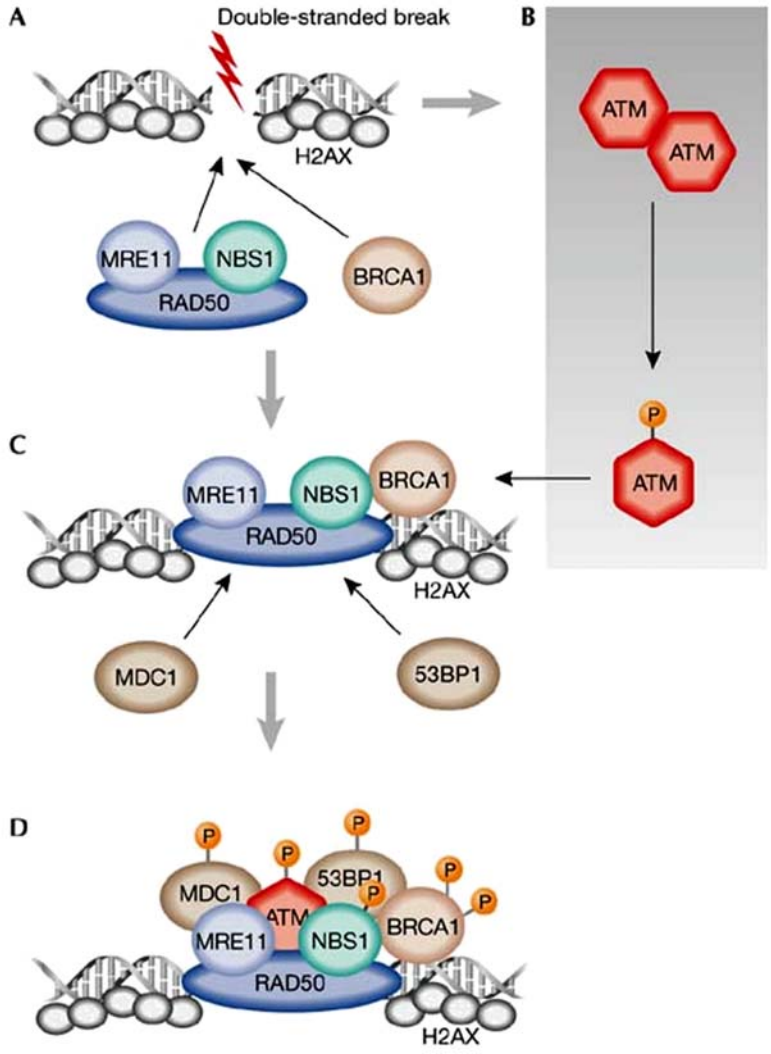

Figure 8: ATMs activity at the double strand break site. (A) Several simultaneous events occur to ultimately activate ATM signal transduction. ATM undergoes auto-phosphorylation to an active monomer (B) A histone variant, histone H2AX, present within chromatin, becomes phosphorylated and serves as a tethering platform for repair factors. The MRE11–RAD50–NBS1 complex locates to the DNA lesion together with BRCA1 (C) Assembly of this complex facilitates coordinated co-localization of active ATM together with other factors including MDC1and 53BP1. BRCA1, MDC1 and 53BP1 are also phosphorylated in an ATM dependent manner (D) The assembly of this multiprotein complex facilitates the cellular response to a DNA double-stranded break (McKinnon, 2004).

When a double strand break occurs, there are two different repair mechanisms that can be initiated: non-homologous end-joining (NHEJ) or homologous recombination (HR) (Figure 9). NHEJ is the most predominant in mammalian cells. This mechanism quickly seals the breaks at the cost of creating inaccurately repaired DNA with microdeletions. The HR

mechanism is much more accurate than the NHEJ mechanism and leaves a completely and accurately repaired DNA. HR is a highly structured process involving several protein complexes. HR is sister chromatid dependent, meaning that HR is only possible in the G2 phase of the cell cycle.

Many proteins and protein complexes are involved in both of the mechanisms.
In the NHEJ mechanism, the KU70-KU80 heterodimer detects the exposed DNA ends and recruits the catalytic subunit of the DNA-dependant protein kinase (DNA-PKcs). The DNA-PKcs is thought to recruit additional proteins to the DNA break. The Ligase IV-XRCC4 complex finally seals the gap (Shiloh, 2003).

The MRN complex, consisting of the MRE11, RAD50 and NBS1 units, is thought to be responsible for carrying out the initial processing of the DSB ends in the HR pathway (Tauchi *et al*., 2002). MRE11 is a nuclease, Rad 50 is an ATPase and NBS1 is a protein responsible for assembling the complex. The MRN complex is also involved in meiotic recombination, telomere maintenance and checkpoint signaling (Shiloh, 2003). The important inter-relationship between ATM and the MRN complex is underscored by the similarity of two other syndromes related to AT that result from hypomorphic mutations in NBS1 and MRE11: Nijmegen breakage syndrome and AT-like disorder (McKinnon, 2004)

In the HR mechanism the RAD51-BRCA2 complex is crucial for strand invasion and strand displacement. This process allows the use of the undamaged sister chromatid as a template for resynthesis of the damaged DNA strands.

Defects in genes coding for any of these proteins may lead to an incomplete DSB response and increased risk of genomic instability

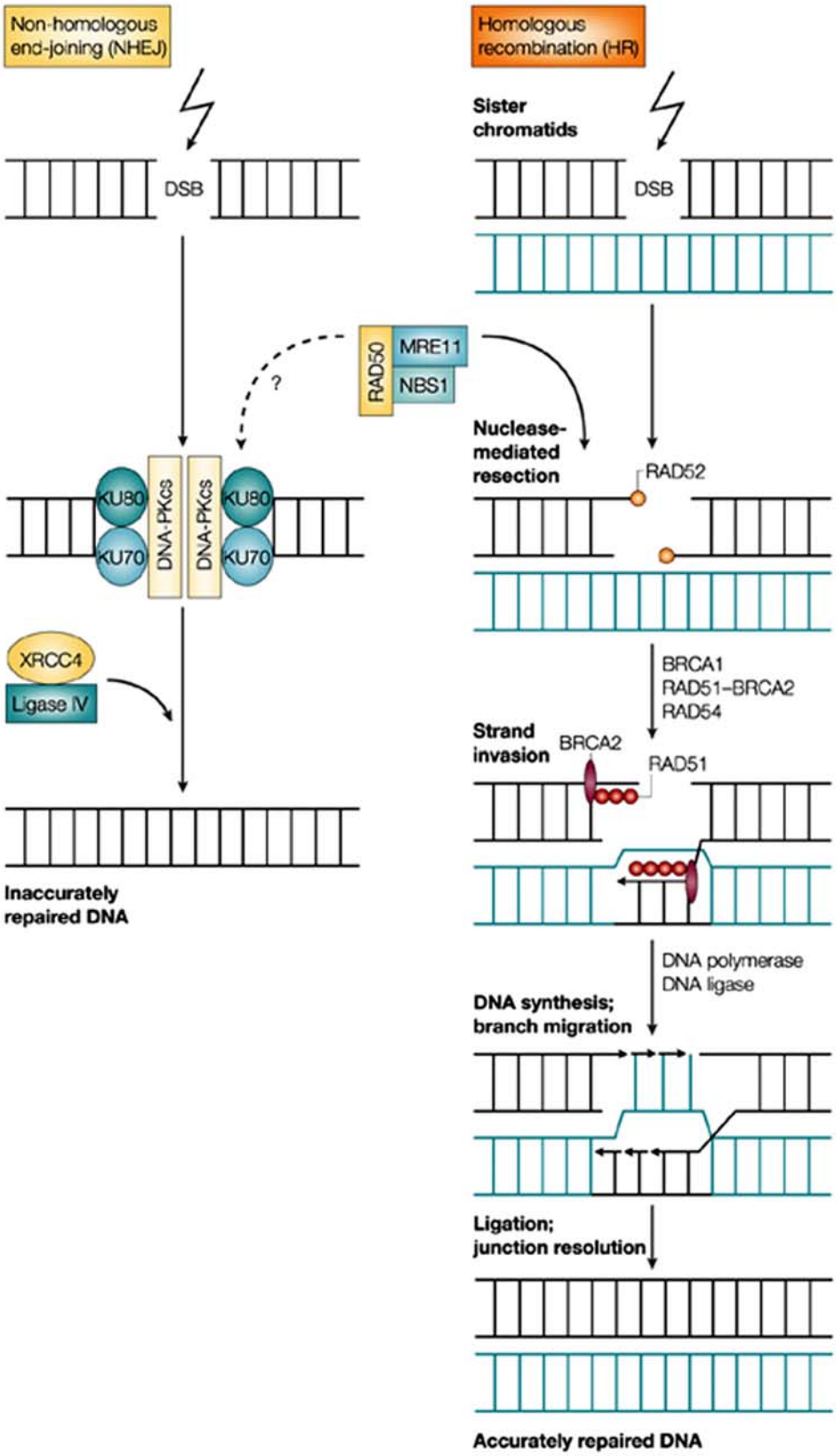

Figure 9: The two mechanisms for repair of DNA double-strand breaks. (Shiloh, 2003)

Activation of ATM will, in a cell with wildtype ATM, lead to cell cycle arrest or apoptosis (Figure 3) and therefore prevent erroneous DNA to be passed on to a new generation of cells. As well as responding to DSBs caused by environmental stress, ATM is also required for processing the physiological DNA strand- breaks that occur during meiosis, immune system maturation and for telomere maintenance (McKinnon, 2004). *ATM* controls processes involving large portions of chromosomes, in response to DSBs or in mitotic recombination and chromosomal segregation (Vogelstein and Kinzler, 2004) and has been classified as a stability gene, or caretaker of the genome.

The simplest interpretation for the role of ATM in preventing AT is that it ensures an appropriate response to DNA damage. This aspect of ATM function explains the immune-system defects that require gene rearrangements for immune maturation, and also the development of lymphoma or leukaemia. Radiosensitivity is also clearly linked to a defective DNA damage response, and sterility results from defects early in meiosis that involves genetic recombination events (Barlow *et al*, 1997). However, some features, such as ocular telangiectasia and insulin resistance, are more difficult to reconcile with a defective DNA damage response (McKinnon, 2004).

ATM mutations

Numerous different mutations have been identified in AT patients. Mutations are found throughout the gene in all 66 exons, with no "hot spots", and seventy percent of the mutations result in a truncated protein (Gatti *et al*., 2001). Milder versions of AT are caused by mutations that result in the production of decreased amounts of functional protein, or normal amounts with markedly reduced kinase activity (McKinnon, 2004).

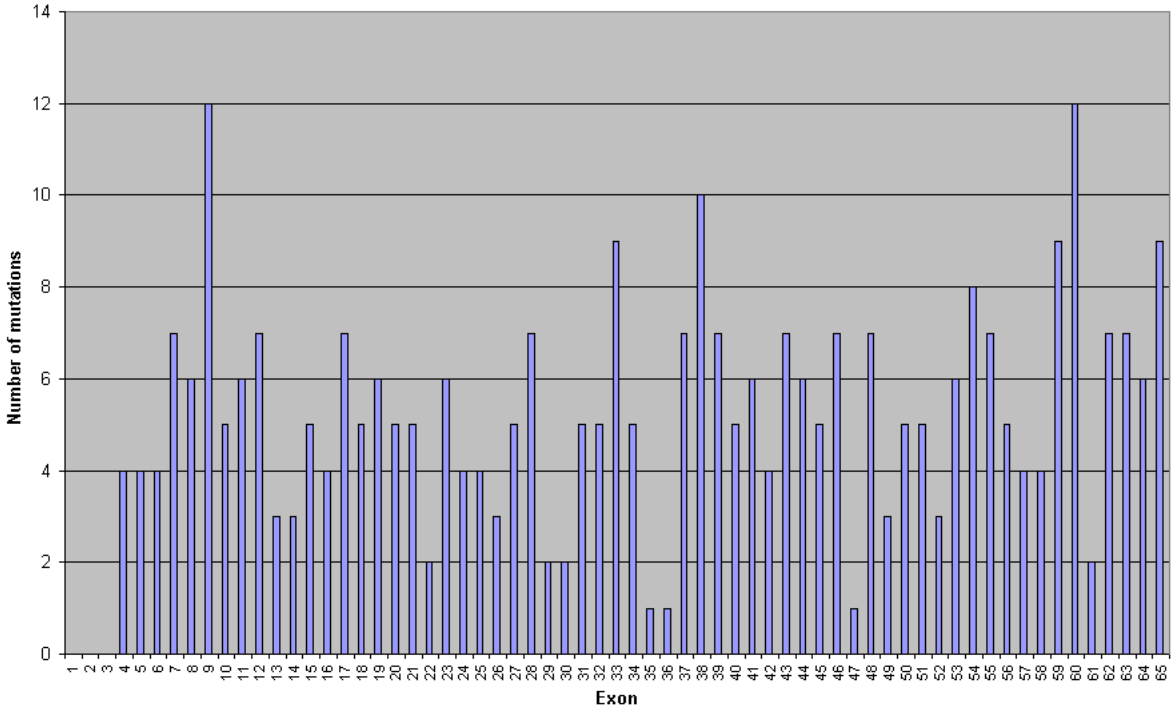

Figure 10: Mutations in the *ATM* gene detected in AT patients by exon[e]

Recurrent mutations are reported in Norway, the Netherlands, Costa Rica, the English Midlands, Italy, Japan, Poland, and among people of Irish English, Utah Mormon and Amish/Mennonite descendance (Laake *et al*., 1998, Gilad *et al*., 1996; Ejima and Sasaki, 1998; Sasaki *et al*., 1998; Stankovic *et al*., 1998; Telatar *et al*., 1998). Most patients have inherited a different mutation from each parent and are namely called compound heterozygotes. The distribution of mutations in the Nordic AT patients is shown in figure 11.

[e] ATM mutation database, http://www.benaroyaresearch.org/investigators/concannon_patrick/images, Accessed 15. January 2005

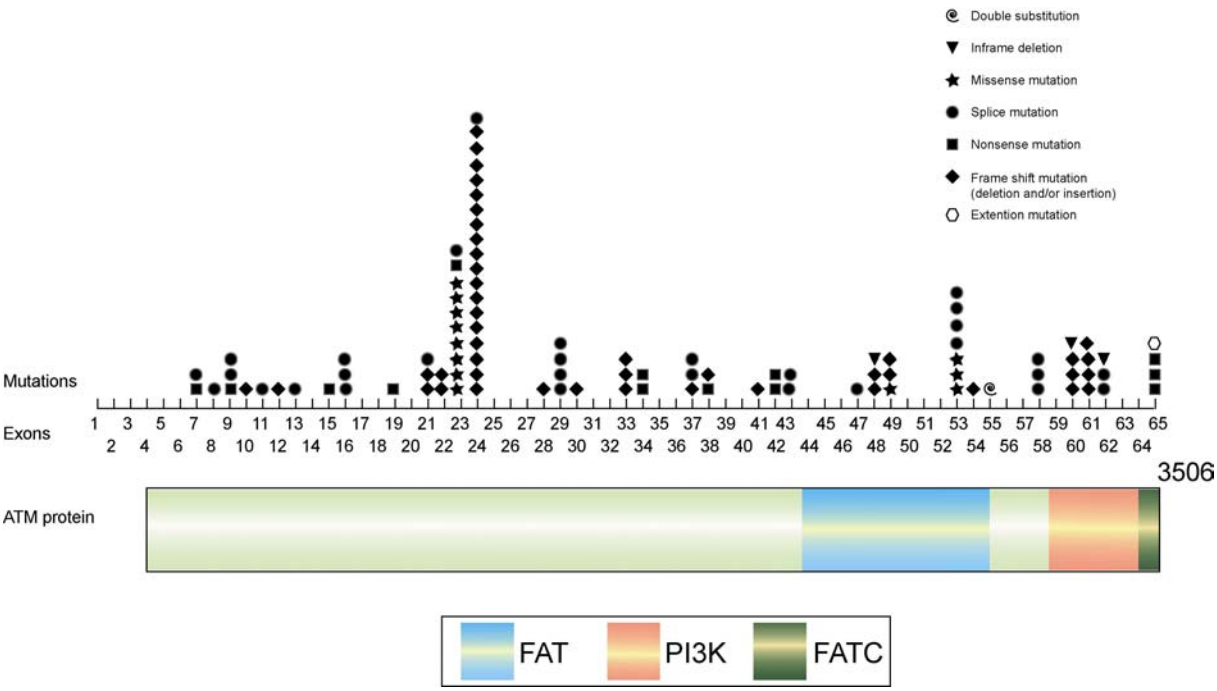

Figure 11: Spectrum of mutations in the Nordic AT patients. Each mutation is represented by a symbol showings its consequence to the transcripts. Mutations in exonic or flanking intronic sequences are located in the nearest exon in the ATM transcript. Mutations found more than once are placed on top of each other, whereas the unique mutations are placed beside each other. Note that in exon 24 there is a Norwegian founder mutation constituting 17 alleles. (Modified from Shiloh, 2003 and Laake *et al*., 2000)

The ATM Mutation Database includes over 300 unique mutations in the ATM gene found in AT probands[f]. The large size of the *ATM* gene together with the diversity and broad distribution of mutations in AT patients has limited the utility of direct mutation screening as a diagnostic tool or as a method of carrier identification, except where founder effect mutations are involved (Concannon and Gatti, 1997).

[f] ATM mutation database, http://www.benaroyaresearch.org/investigators/concannon_patrick/atm.htm, Accessed January 16, 2005

ATM heterozygotes and cancer

Epidemiological studies

Since 0.5%-1% of the population are estimated *ATM* mutation carriers, any increased risk of cancer associated with *ATM* carrier status is of public health relevance. In *ATM* heterozygotes, the relative risk of cancer has been estimated to be 3.8 and 3.5 for men and women, respectively (Swift *et al*., 1991). A summary of some studies on cancer risk among AT relatives is given in table 1.

Table 1: Epidemiological studies of cancer risk in *ATM* heterozygotes

Population	No. of families	No. of relatives	Cancer incidence rate	p-value	Reference
US	44	574	**3.9**[a]	< 0.001	Morrell *et al*., 1990
US	161	1599	**3.8**, men[b]	< 0.05	Swift *et al*., 1991
			3.5, women[b]	< 0.05	
			3.0, men<60[c]	< 0.05	
			2.6, women<60[c]	< 0.05	
Nordic	50	1218	**1.19**[d]	< 0.05	Olsen *et al*., 2001

Boldface numbers are statistically significant

[a] Blood relatives compared with spouse controls

[b] Heterozygous for ATM mutation

[c] Mortality rate for all causes

[d] Standardized incidence ratio of cancer

Increased risk of breast cancer is clearly associated with *ATM* heterozyogosity in females. The risk of dying because of breast cancer is significantly higher for mothers of AT probands. (Swift et al., 1987; Pippard et al., 1988). Table 2 gives a summary of studies on breast cancer and the relative risks (RR) in the most updated reports.

Table 2: Epidemiological studies of breast cancer risk in female AT relatives

Population	No. of families	No. of relatives	No. of breast cancer cases observed	Breast cancer risk	95 % CI	References
US	99	775	25[c]	**3.8**	**1.7-8,4**	Swift *et al*. , 1987; Swift *et al.,*
			21, age <60[c]	**2.9**	**1.1-7.6**	1991; Athma *et al*., 1996
			12, age >60[c]	**6.4**	**1.4-28.8**	
UK	95	414	6[a]	1.7	0.7-4.3	Pippard *et al*., 1998; Easton, 1994;
			3[a,b]	3.4	0.7-9.8	Inskip *et al*., 1999
France	34	1423	9[c]	**4.0**	**1.8-7.5**	Geoffroy-Perez *et al*., 2001
			5 < 45 yrs[c]	**8.4**	**2.7-19.8**	
			4 > 45 yrs[c]	2.4	0.6-6.1	
Nordic countries	66	1448	33	**1.7**	**1.2-2.4**	Olsen *et al*., submitted
			6, age <55[b]	**8.1**	**3.3-17**	
			1, age>55[b]	3.0	0.0-16	

Boldface numbers are statistically significant

[a] Breast cancer deaths

[b] Mothers of AT patients

[c] Obligate ATM heterozygotes (haplotyped/genotyped)

In a study among blood relatives, women with breast cancer were more likely to have been exposed to selected sources of ionizing radiation than controls without cancer, suggesting that diagnostic or occupational exposure to ionizing radiation increases the risk of breast cancer in women heterozygous for *ATM* (Swift et al., 1991).

Almost seven percent of all breast cancers in the U.S. may occur in women who are AT heterozygotes, which is several times greater than the estimated proportion of carriers of *BRCA1* mutations in breast cancer cases with onset at any age (Athma et al., 1996). The increased risk for breast cancer for AT family members has been most evident among younger women, leading to an age-specific relative risk model. This model predicted that 8% of breast cancers in women under age 40 arise in *ATM* carriers, compared with 2% of cases between 40 and 59 years (Easton, 1994).

Molecular studies

The epidemiological studies on AT families indicate an increased risk of cancer among *ATM* heterozygotes, in particular breast cancer among females. Since the *ATM* gene was isolated (Savitsky *et al*., 1995a), breast cancer patients and controls have been screened for *ATM* mutations to evaluate the public health implications on the use of different diagnostic tools and treatment schemes, such as mammographic screening or radiation therapy. However, many of the first case-control studies failed to find significant differences between these two groups based on the frequency of *ATM* mutations (Vorechovsky *et al*, 1996a, 1996b; FitzGerald *et al*., 1997; Chen *et al*., 1998) leading to a discrepancy between these studies and the epidemiological studies. Retrospectively, however, some later studies show an agreement between the previous epidemiological and molecular studies based upon reanalysis of their data (Bishop and Hopper, 1997). Table 3 gives a summary of some of the largest studies of breast cancers and *ATM* mutations.

Table 3: *ATM* mutations in breast cancer patients

Reference	Material	Method	Truncating mutation(s)/ patients screened	Missense substitution(s)/ alleles screened
Vorechovsky *et al*., 1996a	Tumours from unselected BC cases	SSCP	0/38	5/76[a]
Vorechovsky *et al*., 1996b	BC cases in multiple cancer families	SSCP	3/88	12/176
Appleby *et al*., 1997	BC cases with ARRT	REF	0/23	11/46[c]
Fitzgerald *et al.*, 1997	BC cases < 40 years	PTT	2/401 patients 2/202 controls	NA
Chen *et al*., 1998	BC cases from BC families	PTT	1/100	NA
Larson *et al*., 1998	Sib pairs with BC	REC, FA	NA	2/66 sibpairs[d]
Shayeghi *et al*., 1998	BC cases with ARRT	CSGE	1/41	29/82[e]
	BC cases without ARRT		0/38	32/78[e]
Bebb *et al*., 1999	Late-onset sporadic BC cases	PTT	0/48	NA
Izatt *et al*., 1999	BC cases > 40 years	SRA	0/100	36/200[f]
Oppitz *et al*., 1999	BC cases with ARRT	SSCP	0/11	1/22
Broeks *et al*., 2000	BC cases < 45 years, 33 with bilateral cancer	PTT	7/82[g]	NA
Dork *et al*., 2002	BC cases	SSCP	3/1000	460/1000[h]
Thorstenson *et al*., 2003	HBOC families	DHPLC	10/270[h]	NA

ARRT= adverse reactions to radiation therapy, BC= breast cancer, HBOC= hereditary breast and ovarian cancer families, HD=heteroduplex analysis, FA=fragment analysis, NA=not analysed, PTT=protein truncation test, REC=restriction enzyme cutting, REF= restriction fragment polymorphism, DHPLC= denaturing high-pressure liquid cromatography, RT=radiation treated, SRA= sequence restriction assay, SSCP=single strand conformation polymorphism, yPTT=yeast based PTT.

[a] Also seen in the corresponding germline sample (4/4 available cases)

[b] The proband had at least one additional case of breast cancer, lymphoma, leukemia or gastric cancer in her family

[c] One polymorphism in exon 24 of the ATM gene (P1055R) and the presence of a rare HRAS1 allele were analysed in 66 sib pairs. Breast cancer was associated with the presence of the two rare alleles with an odds ratio of 6.9 (95% CI 1.2-38.3)

[d] One commonly detected substitution

[e] 27 of 36 alleles carried a commonly detected substitution (D1853N)

[f] 2 of the alleles carried a commonly detected substitution (D1853N)

[g] The splice mutation, IVS-6 T>G was found in three BC cases and has later been found to be an AT causing mutation (Dork *et al.*, 2002)

[h] Approximately 1 in 50 german BC cases is a carrier of a disease causing *ATM* mutation, in addition a large variety of distinct *ATM* mutations and variants were found and some were significantly associated with BC

[h] Mutations presumed to be disease causing detected in 10 families, both truncating and missense

After many of the early studies failed to find an increased incidence of truncating *ATM* mutations in breast cancer cases and as more sensitive methods for detecting mutations were developed, focus shifted from merely identifying truncating mutations to detecting all types of mutations in the *ATM* gene. In the early studies on breast cancer patients it was noted that missense mutations were detected more frequently than truncating mutations than would have been expected based on the profiles found in AT families. This discrepancy between the findings of familial studies and those of case-control studies suggests that the two types of *ATM* heterozygosity (i.e., the presence of truncating or missense mutations) infer different cancer risks (McConville *et al*., 1996, Gatti *et al*., 1999, Meyn, 1999), with missense mutations associated with an increased risk of cancer and truncating mutations predominantly leading to an AT phenotype. Therefore, some later studies have focused on specific variants in the *ATM* gene in breast cancer cohorts and others on screening the entire *ATM* gene for variants in breast cancer cohorts. Some of these studies are shown in table 4.

One study (Broeks *et al*., 2000) on Dutch patients with breast cancer concluded that *ATM* heterozygotes have an approximately 9-fold increased risk of developing a type of breast cancer characterized by frequent bilateral occurrence, early age at onset, and long-term survival. One splice site mutation, IVS10-6T-G, which leads to AT in the homozygous state, is particularly associated with breast cancer both in the general population and in high-risk breast cancer families (Broeks *et al*., 2003). This has also been confirmed in other studies (Dork *et al*., 2002). Haplotype analyses revealed one common ancestor in all Dutch mutation carriers, and the mutation is estimated to be at least 2000 years old and widely distributed across Europe (Broeks *et al*., 2003).

Table 4: Specific alterations in the *ATM* gene in patients with breast cancer

Reference	Material	Mutation/ polymorphism	Odds ratio	95 % CI
Stankovic *et al*., 1998	Two AT-families	7271T→G	**12.7**	**SS[a]**
Chenevix-Trench *et al*., 2002	One multiple - cancer family[b]	7271T→G	**13.7**	**5.1-36.6**
Bretsky *et al*., 2003	428 BC cases 426 controls	L546V[c]	3.35	1.27-8.84[d]
Bernstein *et al*., 2003	638 unilateral BC[e] 511 bilateral BC	7271T→G	NE[f] NMD	NA
	638 unilateral BC 511 bilateral BC	IVS10–6T→G	NE[g] NE[h]	NA
Angele *et al*., 2003	254 BC, 70 ARRT	IVS22-77 T>C IVS48 + 238 C>G G5557A	**1.75[i]**	**1.09-2.81**
Thorstenson *et al*., 2003	270 HBOC	L1420F	**76[j]**	**5–1227**
Szabo *et al*., 2004	961 BC families[k]	7271T→G	ND	NA
		IVS10–6T→G	1.60	0.48-5.35

Boldface numbers show a statistically significant increased odds ratio

BC= breast cancer, HBOC= hereditary breast and ovarian cancer families, SS=statistically significant, NE=not elevated, NMD= no mutation detected, NA=not applicable, ARRT= adverse reaction to radiotherapy

[a] p-value 0.025

[b] all of the case patients with breast cancer were carriers of the T7271G mutation, as was one woman with gastric cancer

[c] only sequence variation overrepresented among all high-stage breast cancer cases out of 20 analyzed ATM missense variants

[d] After correction for multiple comparisons this variant did not attain statistical significance

[e] 1149 women of age <51 not selected on the basis of family history of cancer but part of ongoing study of bilateral breast cancer

[f] detected in one out of 638 (0.2%) women with unilateral breast cancer

[g] detected in eight out of 638 (1.3%) unilateral breast cancer cases

[h] detected in one out of 511 (0.2%) bilateral breast cancer cases

[I] In individuals carrying variants on both ATM alleles

[j] Observed in seven families who were carriers of L1420F only, not *BRCA1*, *BRCA2* or other *ATM* mutations; 65 families were carriers of other *ATM* variants which indicates an association between *ATM* variants and breast cancer

[k] Families with non-BRCA1/BRCA2 breast cancer from diverse regions

The 7271T-->G *ATM* mutation (Stankovic *et al*., 1998; Chenevix-Trench *et al*., 2002) has been shown to be associated with an increased risk of breast cancer in both homozygotes and heterozygotes. There is a less severe AT phenotype in terms of the degree of cerebellar

degeneration. This mutation (7271T-->G) allows expression of full-length ATM protein at a level comparable with that in unaffected individuals. As table 4 shows, these findings have so far not been confirmed by later studies.

Preliminary data on breast cancer patients participating in an ongoing study on bilateral occurrence of breast cancer, stratified with radiation received for a 1st breast cancer show a significantly increased risk of a 2nd cancer (> 4x elevated) in patients with *ATM* mutations leading to conservative amino acid changes (Børressen-Dale, 2005).

So far, the studies on *ATM* mutations in breast cancer patients conclude that a large variety of distinct *ATM* mutations and variants exist among breast cancer cases and there is a growing body of evidence that *ATM* mutations infer increased susceptibility to breast cancer. Some variants are shown to particularly contribute to the etiology and progression of malignancy (Thorstenson *et al*., 2003; Dork *et al*., 2002; Angele *et al*., 2003; Børresen-Dale, 2005).

In humans, different mutation types are proposed to give different cancer risks. Missense mutations are proposed to have the most elevated risk. However, to separate missense mutations that are "pathogenic" and "non-pathogenic", cDNA containing these mutations have to be inserted into an AT cell line and subsequently radiated. They can be distinguished by their capacity to correct the radiosensitive phenotype in AT cells. Pathogenic missense mutations abolish the radiation induced kinase activity of ATM in normal control cells, cause chromosomal instability and reduced viability in irradiated control cells, whereas the less aggressive mutations do not (Scott *et al*., 2002). The mutant ATM is expressed at the same level as endogenous protein, and interference with normal ATM function seems to be by multimerization. While truncating mutations usually lead to synthesis of nonfunction protein, missense mutations lead to synthesis of a normal level of abnormally functioning ATM protein (McConville *et al*., 1996; Gatti *et al*., 1999; Meyn, 1999). Since the ATM protein is held inactive in dimer or multimer forms (Bakkenist and Kastan, 2003), the presence of a defective ATM protein may inhibit activation of the normal protein. A truncated protein has little or none effect on the activity of the ATM protein produced by the normal allele in *ATM* heterozygotes and therefore would have little or no effect on the overall function of the wildtype ATM. In contrast, the protein encoded by a missense-mutated allele could act as a dominant-negative inhibitor of the ATM protein produced by the normal allele. This would result in an alteration in the activity of the DNA damage detection and repair system, in which

ATM is involved, and therefore in an increase in cancer risk (Chenevix-Trench *et al*., 2002; Scott *et al*., 2002). An ATM 7636del9 mutant protein of a heterozygous knock-in mice displayed dominant-negative activity in cellular assays (Spring *et al*., 2002) and the mice developed an abnormally high number of spontaneous tumours, including a substantial number of mammary tumours (Spring *et al*., 2002). Other results of *in vitro* studies using cells heterozygous for *ATM* mutations also support the idea that a dominant-negative interaction occurs between the normal and mutated forms of the ATM protein.

Cell lines carrying missense mutations are on average more radiosensitive than those carrying truncating mutations (Fernet *et al*., 2004). This is in agreement with the dominant-negative interactions involving missense mutations and influencing cells heterozygous for *ATM* mutation's survival. Studies on extended AT families have failed to detect a significant difference in the relative risk of breast cancer or any other type of cancer based on different types of *ATM* mutations (Cavaciuti *et al*., 2005). However, the occurrence of breast cancer may be associated with truncating mutations in regions encoding certain binding domains of the ATM protein (e.g., TP53/BRCA1, -adaptin, and FAT domains). Therefore, the risk of BC may be associated with the alteration of binding domains rather than with the length of the predicted ATM protein.

Detecting mutations in the *ATM* gene has been one of the greatest challenges in estimating cancer risk in *ATM* heterozygous. With more sensitive techniques available such as DHPLC, the association between different *ATM* gene variants and breast cancer will become clearer in the years to come. Expression profiling by microarray techniques may also prove useful in detecting *ATM* heterozygous (Watts *et al*., 2002). Although the defining characteristic of recessive diseases is the absence of a phenotype in heterozygous carriers, the phenotype is detectable, in lymphoblastoid cells from AT carriers, as changes in expression level of many genes. Carriers of the recessive disease may have an "expression phenotype", and this might prove a new approach to the identification of carriers and enhanced understanding of their increased cancer risk. A summary of present knowledge on types of *ATM* mutations and their phenotypes is given in table 5.

Table 5: *ATM* mutations and phenotype

***ATM* mutation category**	**Phenotype**
truncating/truncating	classical AT
truncating/missense	classical AT
missense/missense[a]	mild/variant AT
wt/truncating	increased risk of bc[b]
wt/missense[c]	increased risk of bc
wt/wt	wildtype

[a] Some variants have an effect on splicing or stereofolding of protein in critical domains and give a classical AT phenotype in the homozygous state.

[b] depending on where the truncating mutation is located

[c] Some variants are associated with increased risk of bc, and radiation sensitivity

TP53

Background

The TP53 is probably the most extensively studied molecule in the cancer field (Bos *et al*., 2004). The realization that *TP53* is a common denominator in human cancer has started an avalanche of publications on that field since 1989. Since then there have been over 17,000 publications on *TP53* and over 19,800 tumour-associated mutations in *TP53* have been discovered (Olivier *et al*., 2004), ranging from humans to clams (Vogelstein *et al*., 2000). *TP53* mutations are common in human cancers, about half of the analysed human tumours. Persons that inherit *TP53* mutations, develop cancers (especially breast, sarcoma, adrenal, and brain) with almost 100% penetrance (Vogelstein *et al*., 2004). TP53*,* identified in 1979, was originally thought to be a tumour antigen (Lane and Crawford, 1979). Mutant TP53 was shown to co-operate with KRAS2, an oncogene, to transform normal cells into neoplastic cells, and was therefore for several years believed to be an oncogene. However, its ability to abolish the tumourigenic phenotype when transfected into tumour cell lines as well as its association with hereditary cancers led to reclassification from oncogene to tumour suppressor gene. *TP53* is now considered to be the guardian of genome (Lane, 1992; Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997; Prives and Hall, 1999; Tlsty, 2002), which maintains genomic stability.

The *TP53* and transcripts

The *TP53,* located at chromosome band 17p13.1, consists of 11 exons within approximately 20 kb of genomic DNA (McBride et al., 1986). Exon 1, located 8-10 kb 5' from exon 2, is non-coding. Two promoters have been demonstrated at the 5'-end of the *TP53* gene; the first is located upstream of exon 1, whereas the second, a stronger promoter, is located within the first intron (Reisman *et al*., 1988).

The TP53 protein

The *TP53* encodes a 53 kD (hence the name, tumour protein 53) cell cycle regulatory nuclear phosphoprotein, which consists of 393 amino acids. TP53s pivotal roles in checkpoint control result from its unique biochemical features. It contains at least three domains: (i) The DNA-binding domain consisting of amino acids from about 100 to 300; this proteolysis-resistant core is flanked by (ii) an N-terminal containing a strong transcription activation signal (Vogelstein and Kinzler, 1994) and (iii) a complex C-terminal that houses nuclear localisation sequences, a homotetramerization domain and a putative DNA damage recognition domain (Fei *et al*., 2003)

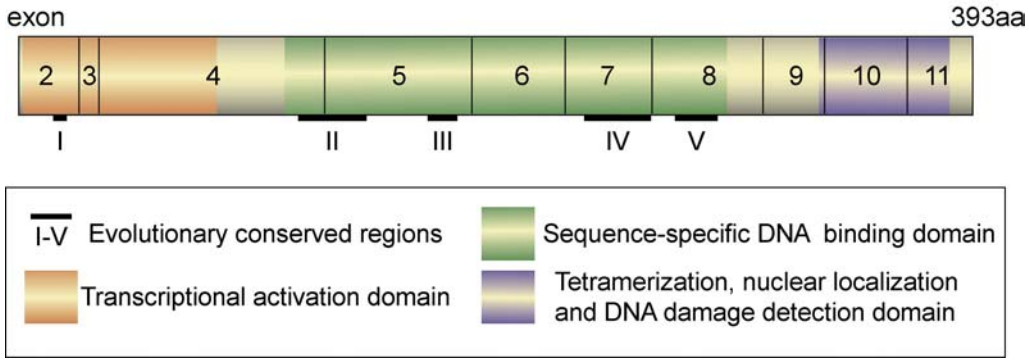

Figure 12: Structural organization of TP53 (Modified from Fei *et al*., 2003)

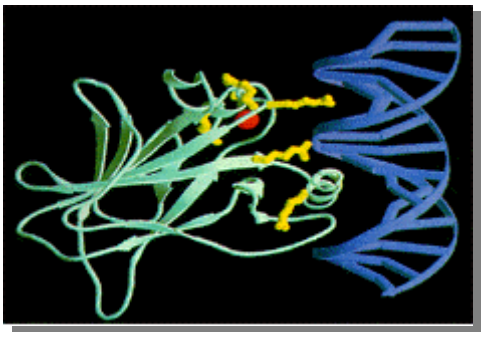

Figure 13: The structure of the core domain of the TP53 bound to DNA (Cho, *et al*., 1994)

Cellular location and stability

The TP53 is primarily located in the nucleus, but it can be detected in the cytoplasm in G1 and following DNA synthesis. Under unstressed circumstances, TP53 is present in a latent state and is maintained at low levels through targeted degradation. Therefore, the protein is undetectable or present at low levels in resting cells, with a half-life of around 20 minutes, being rapidly degraded by the cells proteolytic enzymes. A variety of cellular stresses, such as insults to the cell causing DNA damage, transiently stabilise the TP53, cause it to accumulate in the nucleus, and subsequently activate it as a transcription factor. (Appella and Anderson, 2000). Stressed and damaged cells are more likely than undamaged cells to contain mutations and exhibit abnormal cell cycle control, and therefore present a greater risk of becoming cancerous (Vogelstein and Kinzler, 2004).

Activation - upstream of TP53

The amount and therefore the activity of TP53 is regulated by many different post-translational modifications, including phosphorylation, acetylation, ribosylation, O-glycosylation, ubiquitination and SUMOylation (Fei and El-Deiry, 2003). For example, upon DNA damage, TP53 is phosphorylated at ser15, which induces a conformational change that makes MDM2 unable to bind TP53 and results in the relief of the inhibitory effect of MDM2 on TP53. (Shieh *et al*.,1997). Several important TP53 regulators have emerged, including MDM2, a ring-finger ubiquitin ligase that is both a TP53 target gene and a determinant of TP53 stability; ARF, an inhibitor of MDM2, which increases the stability of TP53; ATM, ATR and CHK1/ CHK2, which can promote TP53 stability by phosphorylating the sites on the TP53 protein that interfere with the binding to MDM2; and several coactivators recruited by TP53 to promote transcriptional activity (Gu *et al*., 1997; Espinosa and Emerson, 2001)

Function – an overview

The TP53 is one of the key proteins in checkpoint pathways, which coordinates DNA repair with cell cycle progression and apoptosis (Fei and El-Deiry, 2003). The TP53 is both a caretaker (by inducing DNA repair) and a gatekeeper (by inducing apoptosis in the case of irreparable damage). When DNA is damaged, TP53 is activated and the necessary responses are induced (Bos *et al*., 2004). The roles of TP53 as a tumour suppressor are predominantly, if not exclusively, mediated through its targets (Fei and El-Deiry, 2003). The TP53 has been shown to be a transcription factor regulating genes that can mediate cell cycle arrest and apoptosis, facilitate DNA repair, or alter other cellular processes (Levine *et al*., 2004). The biochemical function of TP53 that best explains its effects is its sequence specific transcriptional activity, which transactivates target genes through binding a consensus motif in their genomic DNA sequences (Bourdon *et al*., 1997; El-Deiry, 1998; El-Deiry *et al*., 1992; Funk *et al*., 1992). Wildtype TP53 protein has the capacity to both activate and repress gene transcription in order to exert its function in response to genotoxic stress. The function of TP53 is to act as a checkpoint responding to a wide variety of stress signals that can originate from external or internal events (Levine *et al*., 2004). DNA damage, hypoxia, heat shock (denatured proteins), mitotic spindle damage, nucleoside triphossignificance phate pool sizes, nitric oxide signaling, and activation of proto-oncogenes, will all signal to TP53 and elicit a specific response by the cell (Vogelstein *et al*., 2000). TP53 functions as an integrator of the upstream signals and then acts as a central node in a signal transduction network that responds to minimize mutations and other errors that can lead to cancers or other pathologies (Vogelstein *et al*., 2000).

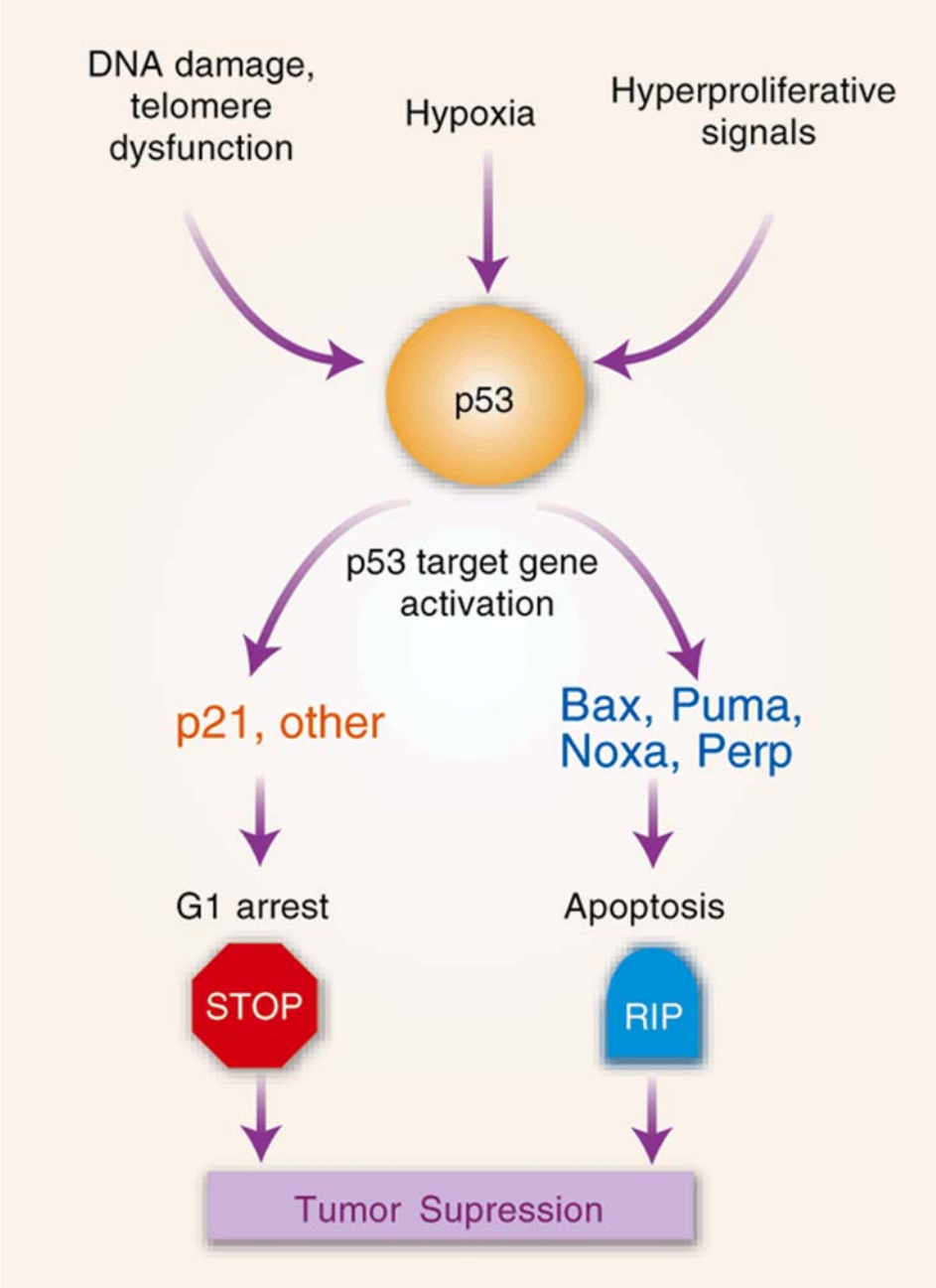

Figure 14: The role of TP53 simplified. A number of cellular stresses, including DNA damage, hypoxia and hyperproliferative signals, activate TP53 to stimulate target gene expression. TP53 induces genes encoding p21 and some other proteins to implement a G1 arrest response and genes encoding Bax, Puma, Noxa and Perp to activate the apoptotic pathway. The particular downstream pathway activated by TP53 is influenced by cellular context, and both pathways contribute to tumour suppression. (Attardi and DePinho, 2004)

Response to the activation of TP53

Several dozen genes that are controlled directly by TP53 have been identified (El-Deiry, 1998), and they fall broadly into four categories based on their effects: cell-cycle inhibition, apoptosis, genetic stability, and prevention of blood vessel formation.

One of the results from TP53 expression is a block in the cell cycle. The TP53 protein induces the expression of p21WAF1/CIP1, an inhibitor of cyclin-dependent kinases (CDKs). CDKs are key regulators of the cell cycle, working together with cyclin proteins to ensure that the genome in the daughter cells are identical to the mother cell. Through its negative effects on various CDKs, p21WAF1/CIP1 inhibits both the G1-to-S and the G2-to-mitosis transitions. (Vogelstein *et al*., 2000)

Another function of TP53 is activation of the apoptotic pathway. There are several potential mediators of TP53-induced apoptosis. The Bax protein is an apoptosis-inducing member of the Bcl-2 protein family. Transcription of the Bax gene in some human cells is directly activated by TP53-binding sites in the regulatory region of the gene. The NOXA and P53AIP1 genes have been shown to be directly activated by TP53 (Oda E *et al*., 2000; Oda K, *et al*., 2000). Like Bax, NOXA and P53AIP1 proteins are located in mitochondria, and when overexpressed, these proteins induce apoptosis. There are other potential mediators of TP53-induced apoptosis such as proteins, which belong to the same family as the TNF (tumour necrosis factor) receptor and Fas. Additionally, TP53 may cause death by directly stimulating mitochondria to produce an excess of highly toxic reactive oxygen species. (Vogelstein *et al*., 2000)

Not all cancer genes are involved in the regulation of the cell cycle, like repair genes, which are involved in repairing DNA damage. Inactivation of such systems may increase the risk of development of cancer. This is because inactivation of these genes leads to an increased risk of mutation in tumour suppressor genes or oncogenes (Vogelstein *et al*., 2000). The TP53 is suggested to be important in maintaining genetic stability, but however, the mechanisms are not clear.

To grow above a certain size, tumours must establish new blood vessels, also called angiogenesis, for transporting nutrients and waste to and from the tumour cells. The wildtype

TP53 stimulates the expression of genes that prevent this process. Cells with inactivated TP53 would therefore be more likely to promote angiogenesis, and therefore provide a critical growth advantage at a late point in tumour development. Different studies confirm that preventing the formation of new blood vessels can be an important component of the activity of a tumour suppressor. The TP53 network is summarized in figure 15.

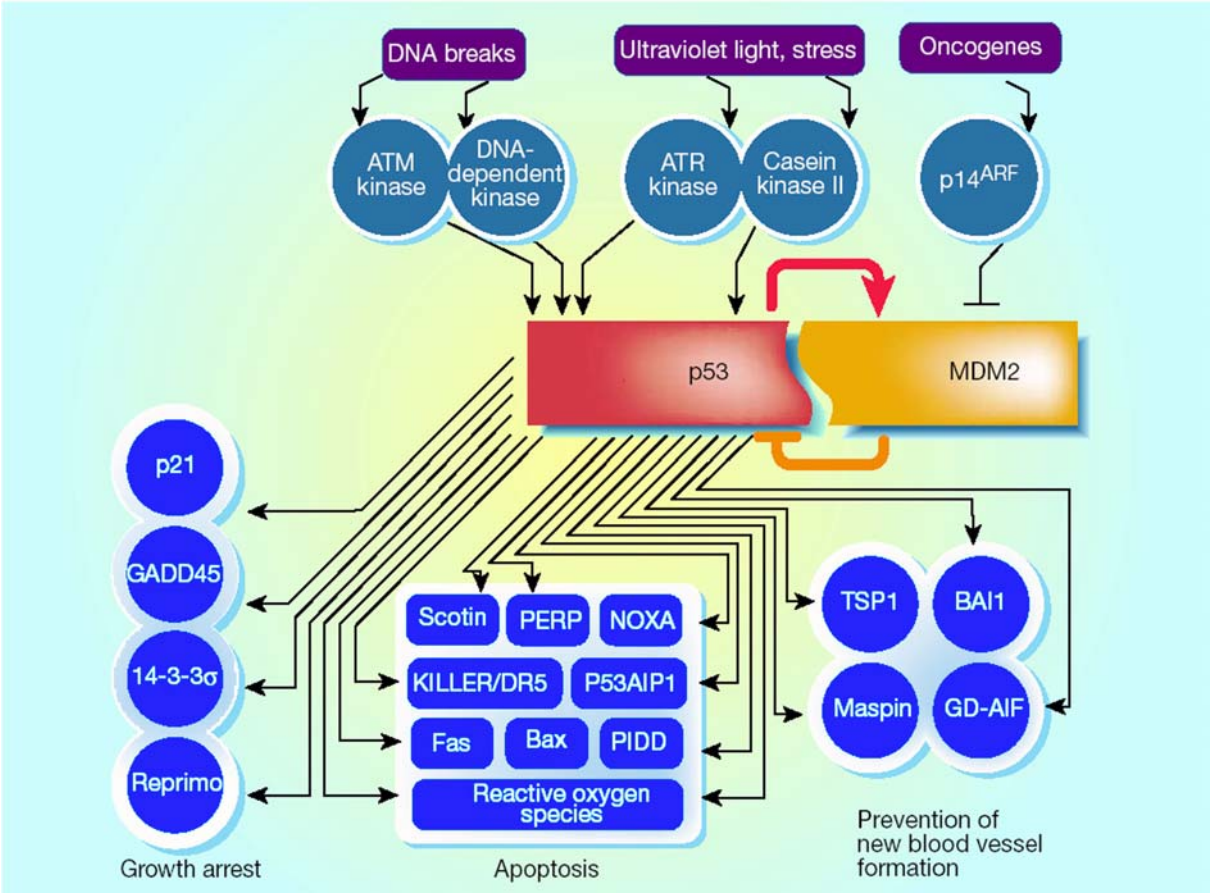

Figure 15: Summary of the TP53 network. TP53 is a highly connected "node" in this network. It is therefore unsurprising that the loss of TP53 function is so damaging, and that such loss occurs in nearly all of human cancers. Many other components of this network, not shown here, have also been identified (Vogelstein *et al*., 2000).

TP53 mutations and cancers

TP53 germline mutation carriers are considered predisposed to cancer since one *TP53* allele is mutated in all cells and only one remaining allele needs to be altered by somatic mutation to be inactivated according to Knudson's two hit hypothesis (Donehower, 1996). In fact, inherited *TP53* mutations are associated with a rare autosomal dominant disorder identified by Li and Fraumeni (1969), called the Li-Fraumeni syndrome (LFS). LFS is characterized by multiple primary neoplasms in children and young adults, with a predominance of soft-tissue sarcomas, osteosarcomas, breast cancer, brain tumors, adrenocortical carcinoma, and leukemia. Up to date, there are 264 described germline mutations in 261 families or individuals (Olivier *et al.*, 2004).

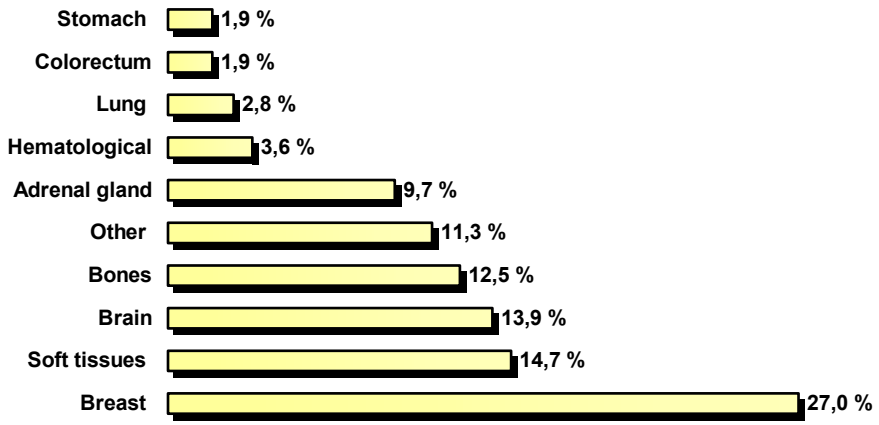

Figure 16: Tumours associated with *TP53* germline mutations (Olivier *et al.*, 2004)

As previously mentioned, somatic *TP53* mutations are found in many tumours. A selection is shown in figure 17.

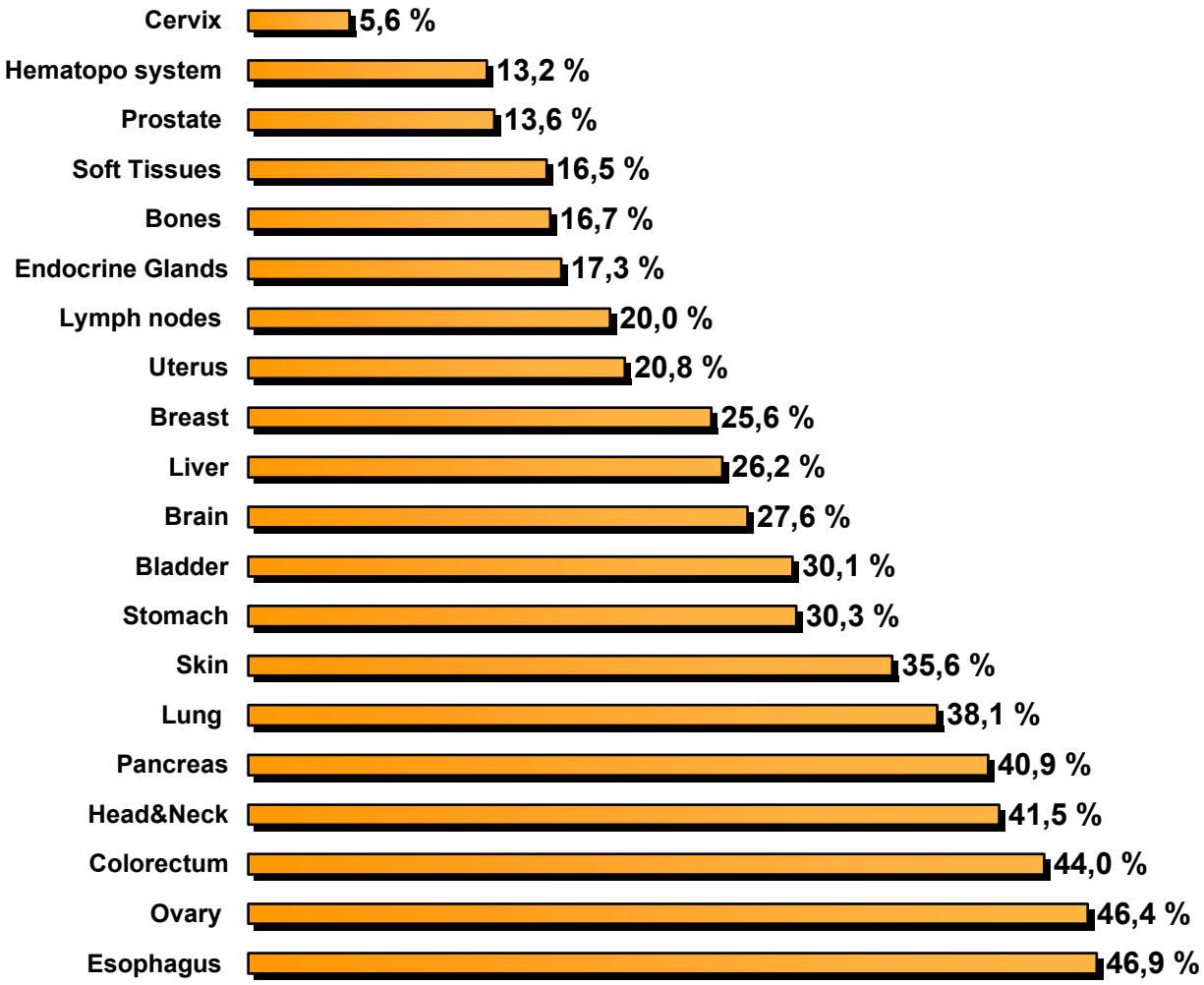

Figure 17: Percentage of *TP53* mutations in different tumour types (Olivier *et al*, 2004)

Almost all of the different mutant TP53s have a longer half-life than the wildtype, which results in an accumulation the protein in the cell. However, the altered protein may functionally differ from the wildtype protein. The majority of mutations found in human tumours are missense mutations, and these mutations are clustered into region encoding the central DNA binding domain (Greenblatt *et al*., 1994) (Figure 18).

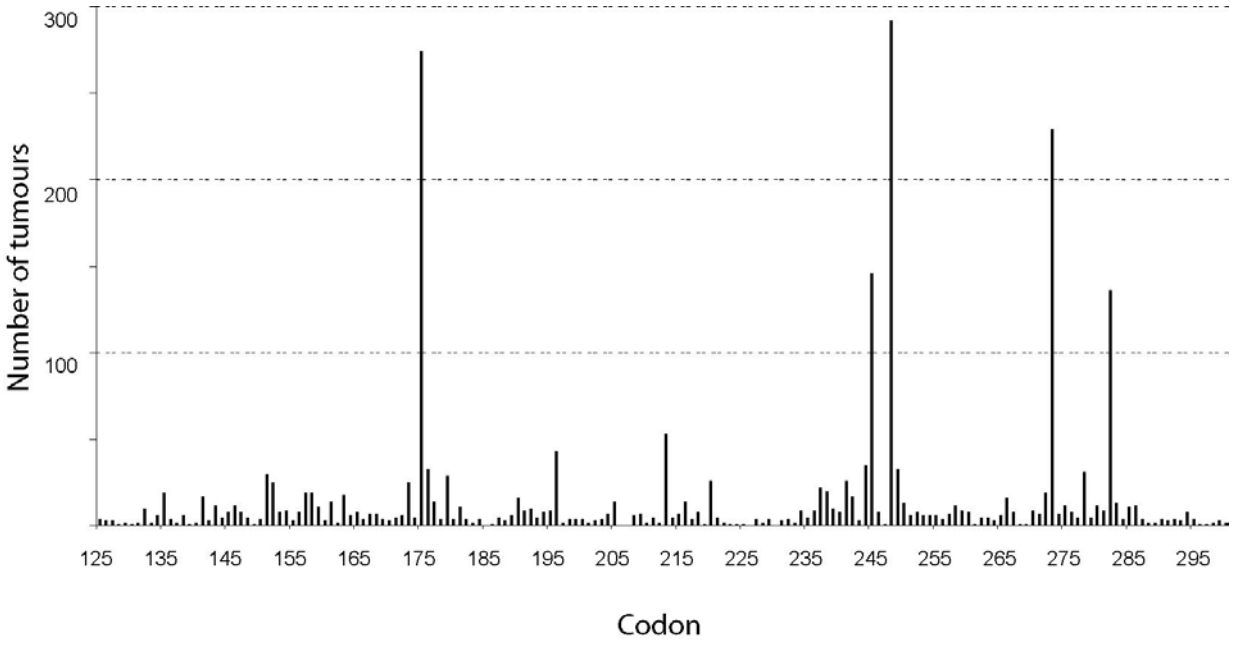

Figure 18: Somatic mutations in *TP53* – distribution of single base substitutions (Olivier *et al*., 2004)

Furthermore, the majority of *TP53* missense mutations, accumulate in cancer and are often retained in distant metastasis. This suggests that a mutation results in more than a loss of function. As previously described for mutated ATM, other properties have also been ascribed to mutant TP53 in addition to a loss of function. These include dominant-negative effects over wild-type TP53 and/or acquisition of new properties (gain-of-function). However, these properties differ from one TP53 mutant protein to another[g].

[g] TP53 mutation database http://www-p53.iarc.fr/p53MUTfunction.html Accessed 20. January 2005

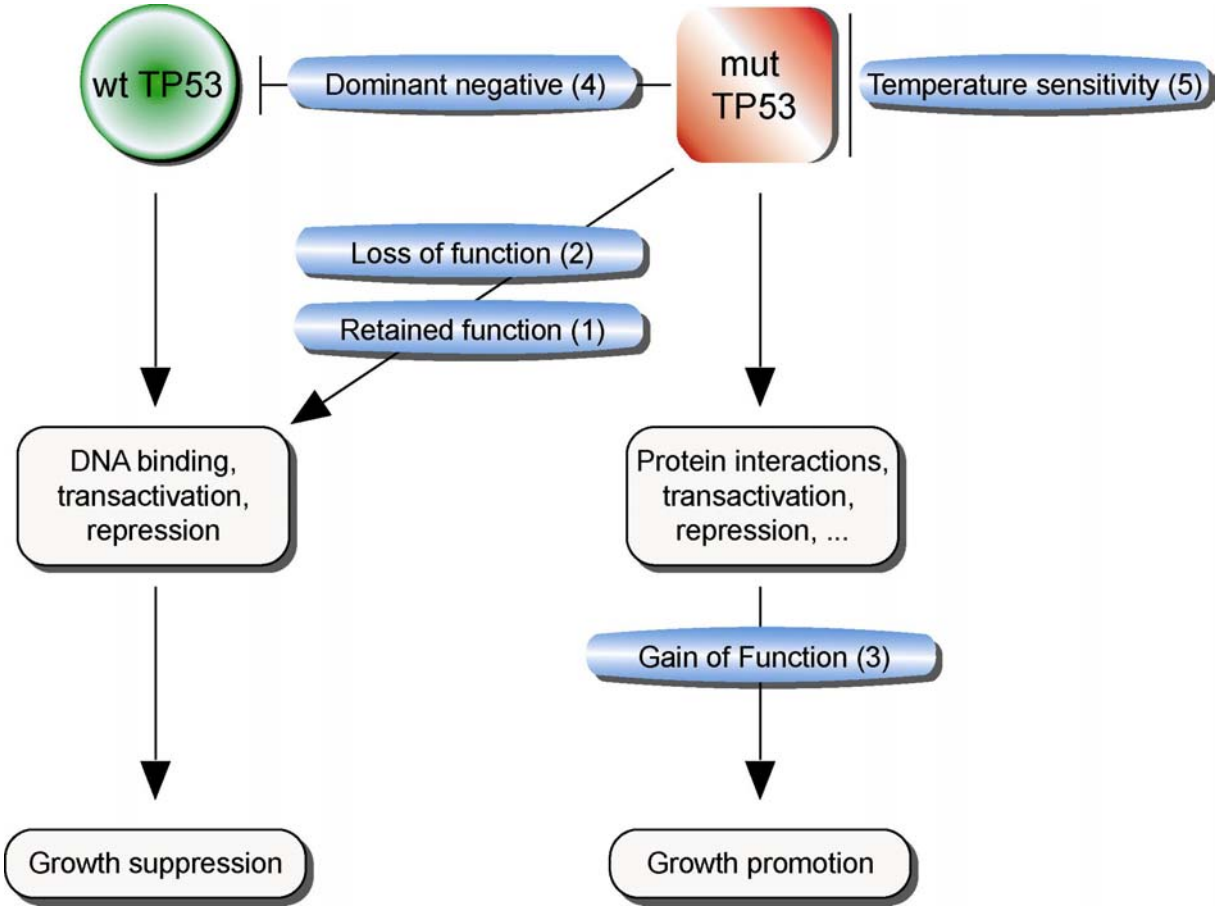

Figure 19: Functional properties of TP53 mutants Functional activities or properties of mutant proteins have been grouped into five categories: (1) retained wild-type activity, (2) loss of function, (3) gain of function, (4) dominant-negative effect, and (5) temperature sensitivity.

While the *TP53* is mutated in approximately 50-55% of cancers, change in TP53 function occurs in an even higher percentage of cancers. In addition to inactivating mutations, TP53 can be inactivated indirectly through binding to viral proteins, or as a result of alterations in genes whose products interact with TP53 or transmit information to or from TP53. Some mechanisms that inactivate *TP53* are listed in Table 6.

Table 6: The many ways in which TP53 may malfunction in human cancers.

Mechanisms of inactivating TP53	Typical tumours	Effect of inactivation
Amino-acid changing mutation in the DNA-binding domain	Colon, breast, lung, bladder, brain, pancreas, stomach, oesophagus and others	Prevents TP53 from binding to specific DNA sequences and activating the adjacent genes
Deletion of the carboxy-terminal domain	Occasional tumours at many different sites	Prevents the formation of tetramers of TP53
Multiplication of the MDM2 gene in the genome	Sarcomas, brain	Extra MDM2 stimulates the degradation of TP53
Viral infection	Cervix, liver, lymphomas	Products of viral oncogenes, bind to and inactivate TP53 in the cell, in some cases stimulating TP53 degradation
Deletion of the $p14^{ARF}$ gene	Breast, brain, lung and others, especially when TP53 itself is not mutated	Failure to inhibit MDM2 and keep TP53 degradation under control
Mislocalisation of TP53 to the cytoplasm, outside the nucleus	Breast, neuroblastomas	Lack of TP53 function (TP53 functions only in the nucleus)

(Modified from Vogelstein *et al*., 2000)

TP53 polymorphisms and cancer

Several DNA polymorphisms in the *TP53* have been identified in human populations[h]. Most reported polymorphisms are localised in introns, outside consensus splicing sites. While these sequence variants theoretically could affect TP53 protein function, the consequences of intronic variations are unclear. An overview of reported polymorphisms is given in table 7.

Table 7: Polymorphisms in *TP53* (Oliver *et al*.,2004)

Exon/ intron	Codon	Nucleotide position[a]	Type[b]	Base sequence in codon	Mutated base sequence	Con-sequence	Prevalence	Reference[c]
Polymorphisms that do not alter the amino acid sequence of p53								
Exon 2	21	11779	Point	GAC	GAT	Silent	unknown	Ahuja *et al*. (1990)
Exon 4	34	12026	Point	CCC	CCA	Silent	20 %	Vos *et al*. (2003)
Exon 4	36	12032	Point	CCG	CCA	Silent	4 %	Felix *et al*. (1994)
Exon 6	213	13399	Point	CGA	CGG	Silent	To 11%	Carbone *et al*. (1991)
Polymorphisms that do alter the amino acid sequence of p53								
Exon 4	47	12063	Point	CCG	TCG	Pro>Ser	To 1.5 %	Felley-Bosco *et al*. (1993)
Exon 4	72	12139	Point	CGC	CCC	Arg>Pro	Wide range Beckman *et a*l. (1994)	Harris *et al*. (1986) Thomas *et al*. (1999)
Polymorphisms in non-coding regions of the TP53 gene[d]								
Intron 3	-	11951	ins	-	-	16bp+	Sjalander *et al*. (1996)	Lazar *et al*. (1993)
Intron 6	-	13494	Point	-	-	G>A	unknown	Chumakov and Jenkins (1991)

[a]Nucleotide position at which the polymorphism is located, based on the GenBank entry X54156.

[b] Mutation-type: point=single base substitution, Ins=insertion.

[c] Reference where the polymorphism has been described for the first time

[d] A total of 15 intronic polymorphisms have been reported to the TP53 mutation database - listed are the most extensively studied

[h] TP53 mutation database: http://www-p53.iarc.fr/Polymorphism.html Accessed 24. January 2005

Only two polymorphisms, proline to serine at residue 47 and arginine to proline at residue 72, alter the amino acid sequence of TP53. The Ser47 variant is a rare polymorphism affecting a codon conserved in evolution. Residue 72, although not conserved, is located within the proline-rich region and may affect the structure of the putative SH3-binding domain. Ethnic differences in codon 72 allele frequencies have been observed. In Western Europe (France, Sweden, and Norway), North America (USA), Central and South America (Mexico, Costa-Rica, Peru) and Japan, the most common allele is Arg72, with frequencies ranging from 0.60 to 0.83.

TP53 protein with Arginine at position 72 is more efficiently targeted for degradation by the E6 protein of HPV16, suggesting that individuals homozygous for Arg72 may be at a higher risk of HPV-related cervical cancers (Storey *et al*., 1998). However, this hypothesis has not been confirmed in human populations (Klug 2001, Koushik 2004). The codon 72 Pro/Pro genotype is associated with an elevated risk of lung cancer (Kawajiri 1993, Wu 2002), however others could not confirm this association (Matakidou 2003). Haplotypes with codon 72 Arg/Pro, intron 6 G>A and intron 3 duplication are associated with increased lung cancer risk and correlated with higher apoptotic indices and DNA repair capacity in lymphoblastoid cell-lines (Wu 2002). The intron 3 duplication gives an increased risk of colorectal cancer and correlates with a reduced level of TP53 mRNA in lymphoblastoid cell-lines (Gemignani 2004). While further studies on these polymorphisms are needed to enlighten their influence on cancer risk, these findings strongly suggest that these polymorphisms may affect TP53 function.

ATM AND TP53 INTERACTION

Both TP53 and ATM have important roles in controlling the integrity of the genome. They function in the same DNA damage response pathway, with ATM as an upstream regulator of TP53 activity. ATM dependent events leading to TP53 stabilisation represent the initial major mechanism by which cells utilize the TP53 pathway for the cellular stress response, leading to cellular growth inhibition and tumor suppression (Bakkenist and Kastan, 2003). However, the presence of multiple positive regulators of TP53 and numerous downstream targets indicates a redundancy that ensures activation of the TP53 pathway (Lozano and Zambretti, 2005). Their common role in inhibiting tumourigenesis is confirmed by the phenotypes of the cells and individuals, homozygous and heterozygote for mutations in *ATM* and *TP53* (Table 8).

Table 8: Comparison of the *ATM* and *TP53* mutated phenotypes (Børresen, 1988)

Phenotype	*ATM*	*TP53*
Cells	No G_1/S and G_2/M arrest after irradiation	No G_1/S arrest after irradiation
Predominant cancer in homozygous	Leukaemia and lymphoma	Leukemia and lymphoma (mice)
Most frequent cancer in heterozygous	Breast	Breast

ATM-deficient cells, have a severe disruption of the G1 checkpoint because the TP53-dependent response to DNA damage is suppressed (Delia *et al*., 2003). While *ATM* heterozygous deficient mice, carrying a knock out null allele, have no increased risk of breast cancer, doubly heterozygous *ATM* and *TP53* deficient mice have a strong enhancement in mammary carcinogenesis following X-ray irradiation (Umesako *et al*., 2005). Thus, when one allele is inactivated in both *ATM* and in *TP53*, the cell is unable to respond properly to radiation and therefore the risk of cancer development increases. These findings indicate that while two "hits" are required for tumourigenesis, these two hits do not have to be in the same gene. This hypothesis is illustrated in figure 20.

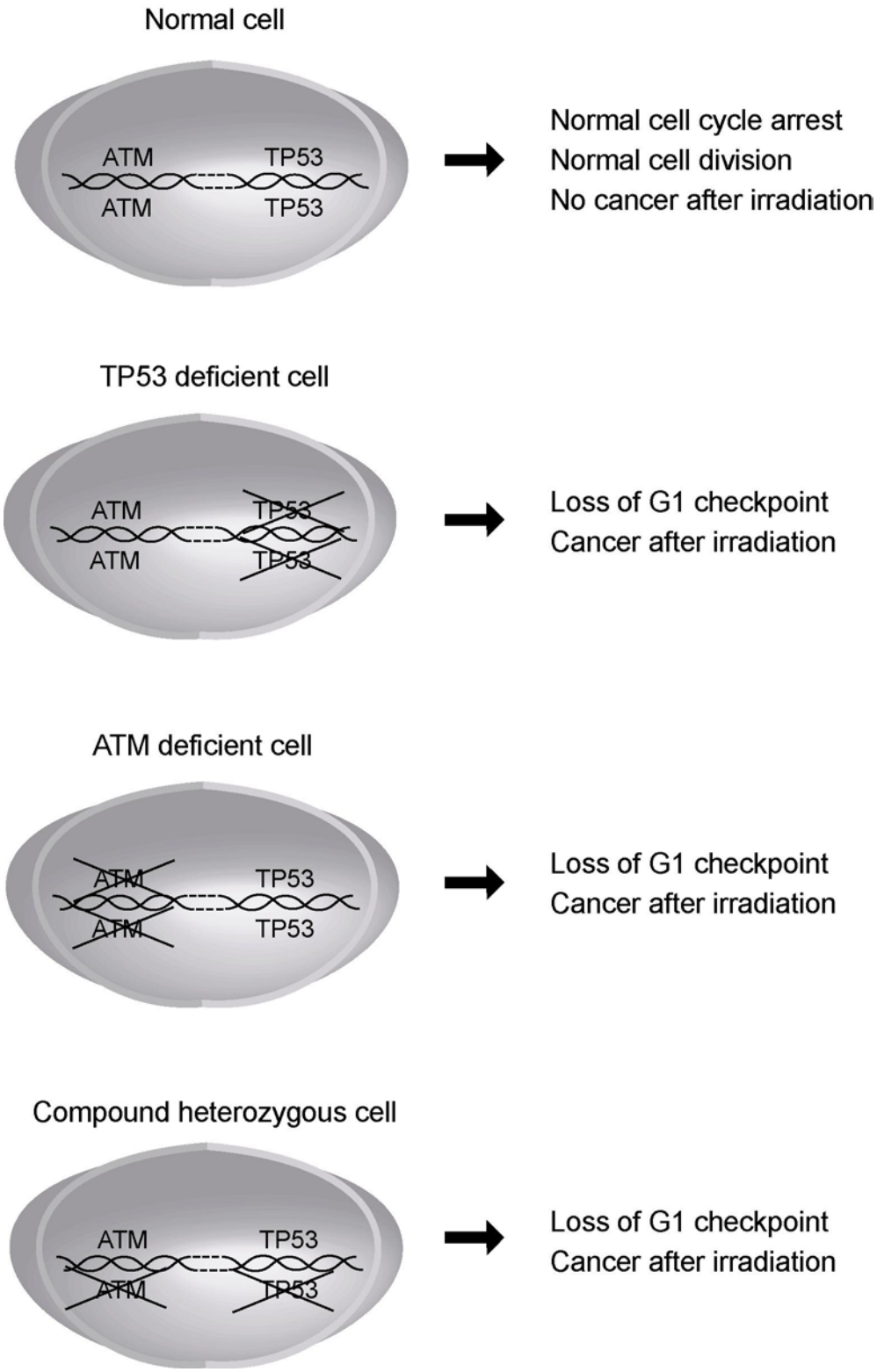

Figure 20: Hypothesis of effects of *ATM* and *TP53* inactivation on cells (Børresen, 1988)

Both mutant ATM and mutant TP53 have demonstrated dominant negative effects over the respective wildtype protein, suppressing the tumour suppressor and caretaker function of the wildtype protein. The inactivation of one allele of both *ATM* and *TP53* is associated with

breast cancer but further studies are needed to enlighten whether specific mutations in *ATM* and *TP53*, separately and in combination infer an even higher cancer risk in humans.

MATERIALS

The Nordic and Norwegian AT study

As a part of the Nordic AT study, pediatric neurologists, pediatric immunologists, medical geneticists, and the medical staff of cytogenetic laboratories and institutions for disabled children were requested to report cases of verified or suspected AT (from 1950 through 1995) to the country's study coordinator. Blood samples, lymphoblastoid cell lines or fibroblasts were available for most families (58 families), either from the proband (or affected siblings) when alive or from the parents. When we were unable to conduct clinical and biochemical examinations to verify the AT cases, the relevant information from the medical records was obtained and reviewed. No case of AT was identified in Iceland (population, 260 000).

For inclusion in the Nordic study the absolute criterion was progressive cerebellar ataxia. Supporting criteria were AT, ocular apraxia, dysarthria, history of infections, elevated alphafetoprotein, chromosome rearrangement involving chromosomes 7 and 14 or increased chromosomal breakage and decreased IgA or IgG2.

A total of 75 patients within 66 families were included, each of whom was characterized by name, sex, and a personal identification number (PIN). The PIN, which incorporates the date of birth, is unique to every Nordic citizen and permits accurate linkage of information among population and health registers. 1445 relatives were identified and relevant cancer diagnoses were obtained from the four countries' cancer registries.

In the Norwegian molecular analysis study, 18 families with a total of 20 probands were included, after informed consents were collected and mutation analysis performed. One family was excluded of own choice and one family was excluded because no AT mutations were found, and the AT diagnosis was not clinically verified. Biological material from probands or their parents was used to identify the disease causing mutation as in the Nordic study. DNA for mutation analysis was extracted from leukocytes in blood, fibroblasts or lymphoblastoid cell lines, using chloroform/phenol extraction followed by ethanol precipitation (Nucleic Acid Extractor 340A; Applied Biosystems) according to standard procedure (Børresen-Dale *et al*., 1998b).

350 relatives of the 18 families were identified, and biological material, blood samples or paraffin embedded tumour tissue was available from 168 blood relatives in the Norwegian AT families. The Norwegian Cancer Registry receives reports of all cancer cases in Norway and paraffin embedded tumour tissue was collected from individuals with a cancer diagnosis. 140 mutation analyses were done on the basis of a blood sample from a live relative and 28 solely on the basis of tissue from an archival tumour block, 13 samples were analysed in both. A description of the paraffin extraction protocol used is found in the appendix.
Tracing of relatives for construction of pedigrees was based on data from the computerized national civil registration systems of Norway, and information on more distant ancestors was derived from manual local population and church registers.

The study on the Pro/Arg polymorphism in mutated *TP53*

The study included 390 Norwegian breast cancer cases. These were from two different consecutive series (129 and 130 samples, respectively) previously described for *TP53* mutation status (Andersen *et al*., 1993; Bukholm *et al*., 1997) and from two series of advanced breast cancer cases (84 and 47, respectively), of which, one had been described previously (Geisler *et al*., 2001). One hundred sixty-two Norwegian colorectal cancer cases previously analyzed for *TP53* mutations in their tumors were also included in this study (Børresen-Dale *et al*., 1998). DNA had been isolated from both blood cells and tumor tissue using chloroform/phenol extraction followed by ethanol precipitation (Nucleic Acid Extractor 340A; Applied Biosystems) according to standard procedure (Børresen-Dale *et al*., 1998b).

METHODS

Polymerase chain reaction

The methods used in this thesis to detect mutations and polymorphisms are based on amplification of DNA by PCR. The PCR is an *in vitro* method for enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA. A cycle of denaturation, annealing (hybridisation of the primer), and extention (synthesis of the DNA fragment) is repeated until a certain amount of product has been produced (figure 21). The synthesis operates in the 3'-end of the primer, towards the other primer, while the 5'-end defines the size of the fragment. During synthesis, a DNA polymerase connects the deoxyribonucleoside triphosphate molecules (dNTP) one by one at the growing 3'-end of the primer. The reaction takes place in a buffer to give the polymerase the best working conditions.

In theory, only one single template is needed, therefore in performing PCR, one has to be particularly observant to problems concerning contamination. A negative control, without template DNA, is important to include in every run to exclude contamination.

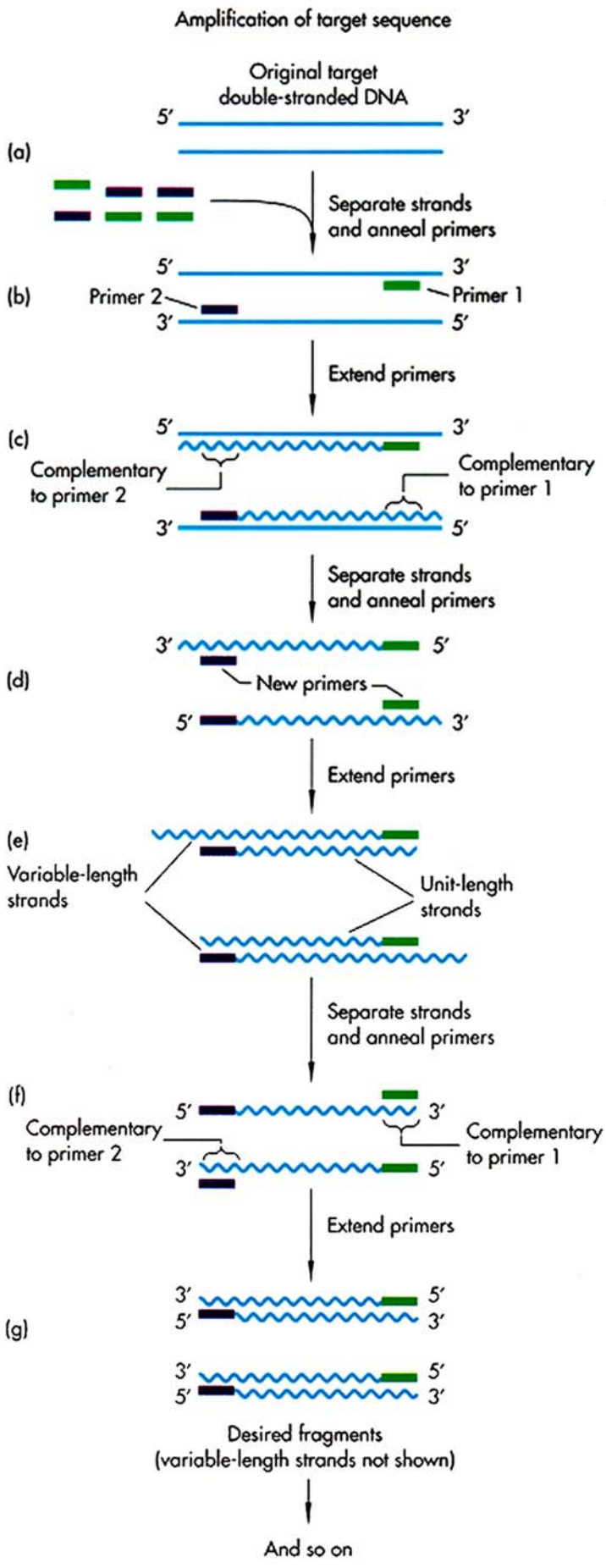

Figure 21: An illustration of the polymerase chain reaction (PCR). (a) The solution is heated to 95°C to denature ("unzip") the two strands of the target DNA (b) The solution is cooled to ~55°C to allow the primers to anneal (bind) to the ends of the DNA strands (c) The solution is reheated to ~72°C to allow Taq polymerase to synthesize complementary copies of each strand (d-g) The cycle is repeated until a certain amount of product has been produced. The products of one cycle are used as template in the next cycle, giving an exponential growth of a given fragment. 20 cycles of PCR produces approximately one million (2^{20}) amplifications.

Heteroduplex analysis

The methods used for identifying mutant *ATM* and *TP53* alleles are based upon heteroduplex formation after PCR. The PCR product is heated to 95°C and slowly cooled to room temparature. The principle of heteroduplex formation is illustrated in figure 22

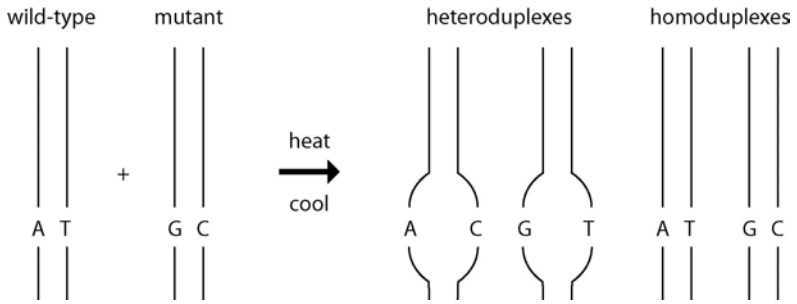

Figure 22: Principles of heteroduplex formation

As a result of the heteroduplex formation caused by mutant alleles, the DNA will behave differently in an electrical field, temperature and chemical gradient. Thus, it becomes possible to separate mutant alleles from wildtype alleles when the DNA is submitted to varying conditions.

DNA sequence analysis

To determine the exact sequence of a mutation detected by heteroduplex analysis, DNA is sequenced in both directions using DyeDeoxy Terminator Cycle sequencing kits (Applied Biosystems) using an ABI 3100 DNA sequencer. In di-deoxy sequencing, DNA polymerase is put together with dNTPs in four different reactions, each containing a small amount of one di-deoxy NTP (ATP, CTP, GTP or TTP). The di-deoxy nucleotide lacks a 3'OH to continue chain extension, so the chain terminates. Each reaction produces a population of fragments terminated at A, T, C, or G. The fragments are labelled with fluorescent dyes and loaded in a capillary tube and subjected to electrophoresis[i]. Sequences of DNA in the gel lanes are read by a computerized fluorescence detection system that measures the intensity of light emission from each "band". The final output is a "trace" or "electrophoretogram" that plots the intensity

[i] Biology Dept Kenyon College http://biology.kenyon.edu/courses/biol114/Chap08/Chapter_08a.html Accessed 1. Februrary 2005

of different color emissions vs. the length of the DNA being sequenced. By observing the progression of peaks of different colors, the DNA sequence is derived (A C G T).

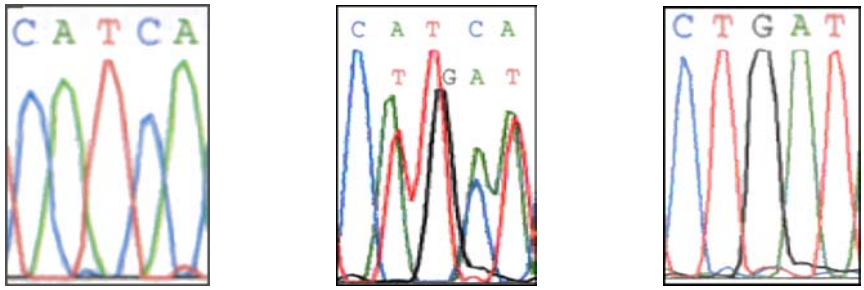

Figure 23: An example of DNA sequencing results of the *ATM* gene, exon 24, codon 1082, showing the wildtype alleles, a heterozygote for the delATC insTGAT mutation, and a homozygote for the delATC insTGAT mutation.

Screening for mutations in AT probands

The Nordic and Norwegian AT family study has been ongoing for several years. Biological samples are continuously coming in and genetic analysis is performed consecutively. Therefore, different methods for mutation analysis, have been used over the years to detect mutations in the probands. The *ATM* gene has been screened for disease-causing mutations by heteroduplex analysis using DHPLC or protein truncating testing with subsequent sequence analysis at the cDNA and genomic level to identify the nature of the mutation.

Denaturing High-Pressure Liquid Cromatgraphy (DHPLC)

DHPLC is the most sensitive method for detecting mutations in the *ATM* gene so far (Bernstein, 2003). The wildtype allele and mutant allele are multiplied, heated and cooled to form heteroduplexes with mismatched basepairs and homoduplexes. The sample is injected into a flow path containing Acetonitrile (ACN) and Triethylammonium Acetate (TEAA). It then passes through a cartridge with hydrophobic beads with temperature set for partially denaturing conditions. The hydrophopic portion of TEAA interacts with the hydrophobic beads in the cartridge, subsequently the negatively charged phosphate backbones of the

partially denatured DNA are attracted to the positively charged ammonium groups of the TEAA. At increasing concentrations of ACN the TEEA/DNA attraction is reduced and the fragments begin to elute off the cartridge. The heteroduplex with the mismatched base pairs elute off the cartridge first. The homoduplexes elute off the cartridge next. As the DNA fragments pass through an UV detector, absorbance is measured. The heteroduplex pass through first, then the homoduplexes. Data from the UV detector are sent to the computer for analysis[j]. X-axis represents time and peaks represent DNA fragments in figure 24.

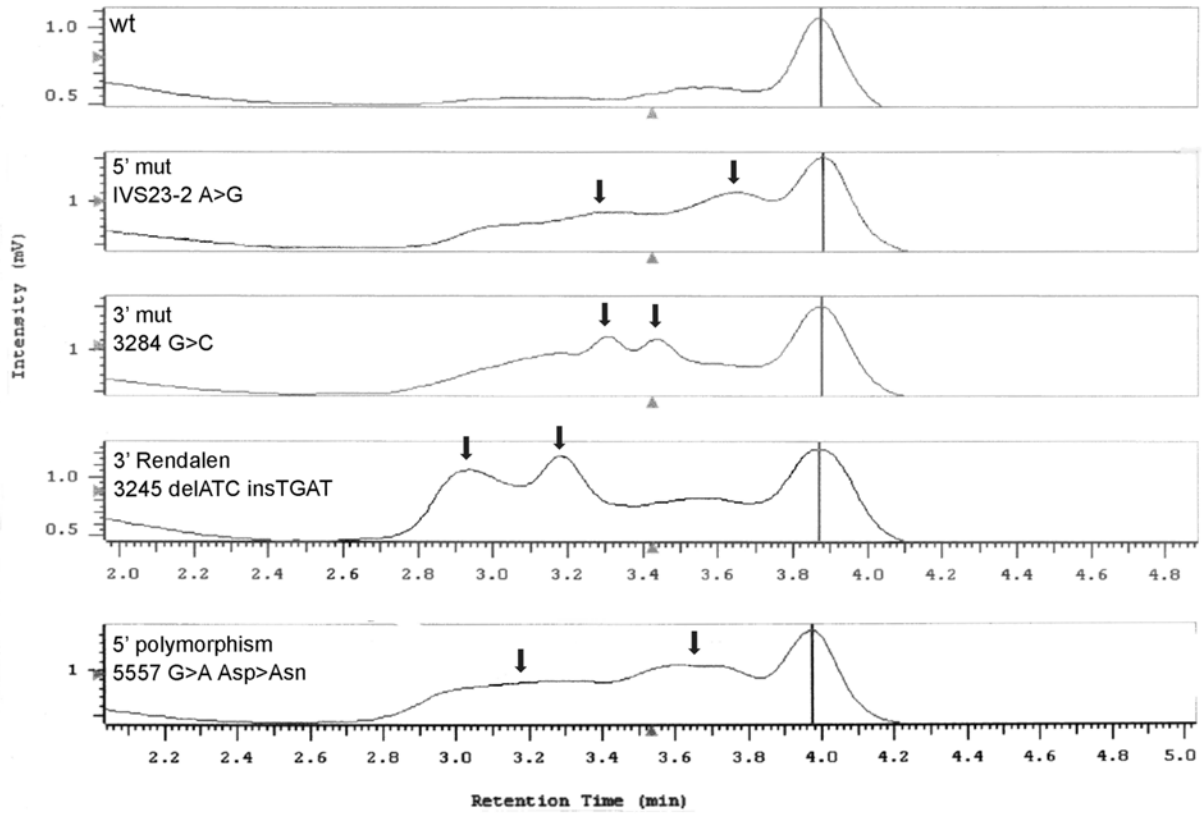

Figure 24: DHPLC screening of exon 24 for *ATM* mutations (top four graphs) and exon 39 (bottom graph) for the most common polymorphism in the *ATM* – experiment conditions are optimalized in order to discover as many genetic variances as possible with the same settings. Cartridge temperature of this experiment was 58°C. Conditions have been described elsewhere (Bernstein *et al*., 2003).

Protein truncation test

The protein truncation test is designed to screen the coding region of a gene for mutations that cause premature termination of translation, large in frame deletions or insertions (reviewed in

[j] Transgenomics http://www.transgenomics.com Accessed 15. January 2005

Den Dunnen and Van Ommen, 1999). The principles of the PTT technique are outlined in figure 25. The PTT technique cannot detect epigenetic changes (like methylation), gross genomic alterations or minor changes not leading to truncated protein.

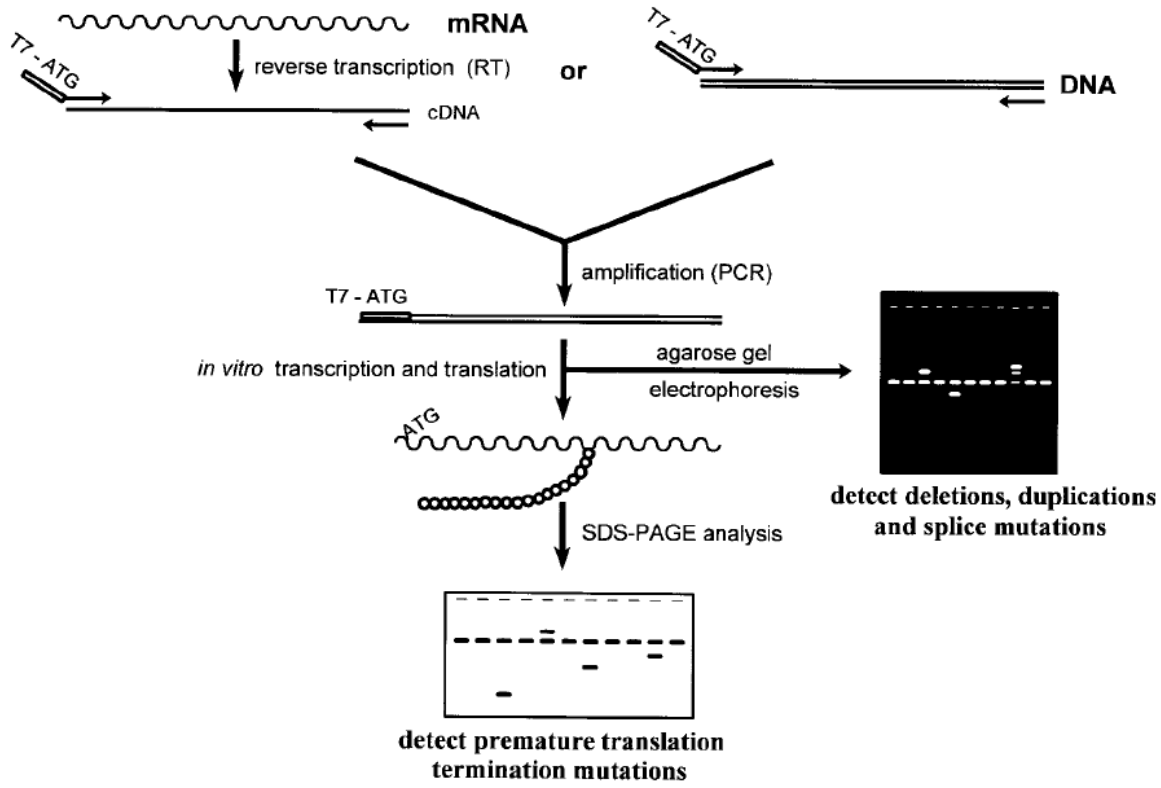

Figure 25: Schematic presentation of the protein truncation test. First, RNA is reverse transcribed (RT) to generate a cDNA copy. Second, the cDNA (or genomic DNA) is amplified using the polymerase chain reaction (PCR) in combination with a specifically tailed forward primer facilitating in vitro transcription by T7-RNA polymerase. Products are analyzed on an agarose gel to verify amplification, determine yield, and check size; abnormally migrating products point to mutations (insertions, deletions, duplications, or affecting splicing). Finally, in vitro transcription/ translation is used to generate peptide fragments, analyzed on SDS-PAGE gel, to detect translation terminating mutations (Den Dunnen and Van Ommen, 1999).

Identifying *ATM* mutation carriers in AT relatives

Once the mutations on both alleles were identified in the proband and the maternal and paternal alleles were designated, mutation analysis of relatives was done by heteroduplex analysis of the known mutation in the corresponding parental branch by electrophoresis on a 7,5 % polyacrylamid gel or by direct sequencing. The gel is stained with ethidium bromide

and then photographed using a Polaroid camera or a video based instrument (Gel Doc 1000, Bio Rad).

The Norwegian founder mutation was detected by restriction enzyme cut, adding the enzyme *Hp*hI, with cutting site in exon 24, for 2 hours in 37°C, to the PCR product. The mutant allele does not contain the restriction cut site and was separated from the digested wildtype allele in the electrophoresis as displayed in figure 26.

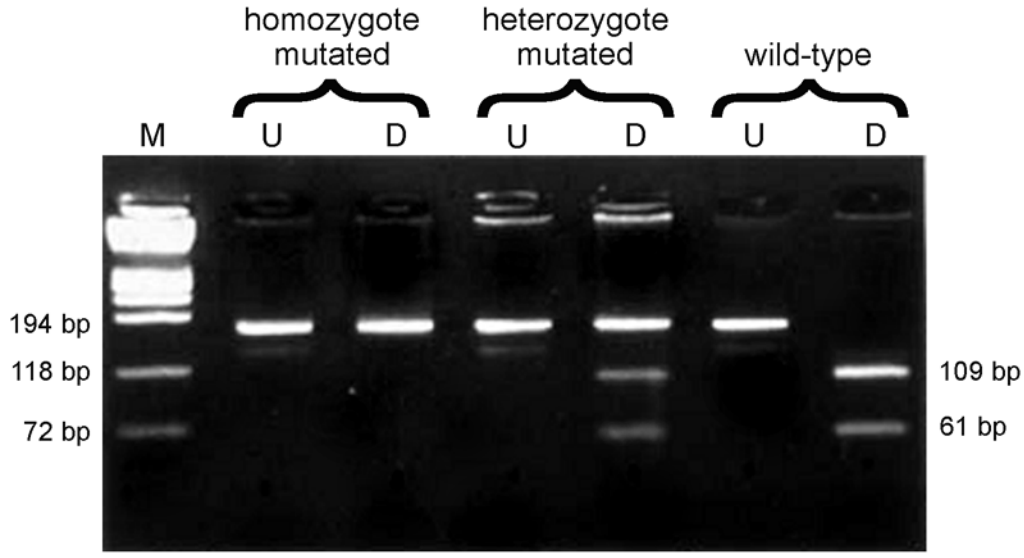

Figure 26: Analysis by electrophoresis of DNA fragment from the *ATM* gene, exon 24, after restriction enzyme cut U=undigested; D=digested; M=size marker; bp=basepairs

Detecting the *TP53* Pro/Arg polymorphism

Restriction fragment length polymorphism analysis

The genetic variation in codon 72 in exon 4 of the *TP53* gene was detected using Restriction Fragment Length Polymorphism analysis (RFLP) (Ara *et al*., 1990). The single-base change that causes an alteration of amino acid residue 72 from arginine to proline, was detected when the PCR product (119 p) was digested with *Bst*UI for 3 hours at 60°C. The DNA fragments were separated by electrophoresis on a 4% agarose gel and gave an allele pattern of the two alleles of 113 bp + 86 bp (Arg) and 119 bp (Pro).

Statistical methods

The Nordic and Norwegian AT study

In the Nordic and the Norwegian study, the standardized incidence ratio (SIR) of cancer was calculated for carriers of an *ATM* mutation. National incidence rates for the tumour categories were calculated according to sex, age (in 5-year groups), and 5-year calendar periods and applied to the person-years of observation in the respective national subcohorts to obtain the number of cancers expected. In the Nordic study the observed and expected numbers of cancers, stratified by type of familial relationship and probability of carrying a mutation in the *ATM* gene, were pooled across country borders, and the SIRs were calculated. In the Norwegian study the observed and expected numbers of cancers in the individuals confirmed heterozygote were used to calculate SIRs. The 95% confidence intervals for the SIRs were calculated by assuming a Poisson distribution of the observed cancers.

The Pro/Arg polymorphism in mutated TP53

Cross-tabulation and χ^2 test were performed when studying the polymorphism's association with *TP53* mutations. Pearson χ^2 test or Fisher's exact test (when appropriate) was used, and statistical significance level was set to $P < 0.05$. Computations were performed using Excel and SPSS.

SUMMARY OF RESULTS

Paper I

The Norwegian AT families were characterized by pedigrees, through genealogical studies, by mutation analysis and calculation of the standardised incidence ratio of cancer for carriers of *ATM* mutations. Seven cancers occurred among the AT probands, 0,06 expected, yielding a standardized incidence ratio (SIR) of 119 (95% confidence interval (CI), 88-181). 168 blood relatives were analysed for the *ATM* mutation found in their family, an 85 were found to be heterozygotes. Among these carriers, 23 cancers were found (SIR 2.2; 95%CI 1.7-3.0). Invasive breast cancer was found in 6 cases (SIR 3.6; 95%CI 2.8-5.0), of whom 3 where mothers of probands. The SIR of breast cancer in females other than mothers of probands was 2.2 (1.6-3.0). One Norwegian founder mutation accounted for 47% of disease causing alleles and there is a high degree of consanguinity in the Norwegian AT population. Carriers of the founder mutation had an elevated cancer risk for all sites, and the highest risk was for breast cancer in females. When considering carriers of other mutations, a slightly higher risk of breast cancer and cancer at all sites was seen except an elevated risk of cancer at all sites in female, although this difference was not statistically significant. When DNA from both blood and tumour tissue from the same individual was analysed, some discrepancies were detected, possibly attributed to the genetic instability in many tumours resulting in loss of one allele.

Paper II

This study was an updated report on a previously published cohort study of cancer incidence in 1218 blood relatives of 56 Nordic AT patients from 50 families (Olsen *et al,* 2001). In this update, 1445 blood relatives, 75 patients in 66 families were included. The *ATM* mutation carrier probabilities of relatives were assigned from the combined information on location in family, consanguinity, if any, and supplementary carrier screening in some families (see paper I). Among the 1445 blood relatives of AT patients, 225 cancers were observed, with 170.4 expected, yielding a standardized incidence ratio (SIR) of 1.3 (95% confidence interval (CI), 1.1–1.4). Invasive breast cancer occurred in 34 female relatives (SIR, 1.7; 95% CI, 1.2–2.4) and was diagnosed in 21 women before the age of 55 (SIR, 2.9; 95% CI, 1.8–4.5) including seven mothers of probands (SIR, 8.1; 95% CI, 3.3–17). In the group of verified *ATM*

heterozygote females, the risk of breast cancer was 2.7 fold increased considering all age groups, and 5.2 fold increased in females below the age of 55. When the group of mothers was excluded, no clear relationship was observed between the allocated mutation carrier probability of each family member and the extent of breast cancer risk.

Paper III

An Arg/Pro polymorphism in codon 72 of the *TP53* gene was analyzed in blood samples from 390 breast and 162 colorectal cancer patients previously investigated for *TP53* mutations in their tumours. Among the breast cancer cases, 228 were homozygous for the Arg72 allele, of which, 65 (28.5%) also had a *TP53* mutation in their tumours. In contrast, of the 26 cases that were homozygous for the Pro72 allele, only 1 case (3.8%) had a *TP53* mutation in the tumour (P =0.004). Cloning the *TP53* gene from tumour DNA followed by sequencing was performed in 14 heterozygotes with tumour mutation, and 9 of the mutations resided on the Arg72 allele. Among the colorectal cancer cases, no difference in mutation frequency was seen between the two different homozygotes, 40 *TP53* mutations in 97 Arg72 homozygous cases (41.2%) *versus* 7 in 16 Pro72 homozygous cases (43.8%).

DISCUSSION OF RESULTS

Cancer risk in *ATM* heterozygotes

Pedigree analyses and mutation detection

In the Norwegian AT population a founder mutation is responsible for 47% of the disease causing alleles; found in 11 of 18 families. A common attendant haplotype has been seen in all individuals carrying the mutation (Laake *et al*., 1998) and five of the families are demonstrated descendants of a couple born in the 15th-century. Most likely there are even more families attached to the "Rendal-pedigree". In families as these, with a high degree of consanguinity and in the presence of a founder mutation, it is essential that epidemiological studies are combined with molecular studies and pedigree analyses. Estimation of carrier probability – especially background probability - would be inaccurate in these Norwegian AT families, since they have a much greater risk of being carriers than the rest of the population. Thus, without molecular analysis it is impossible to reveal a true correlation between carrier status and increased cancer risk.

Breast cancer risk in females

The risk of female breast cancer was 3.6-fold increased in the Norwegian study in all female relatives verified *ATM* heterozygous with a lower limit of the 95% confidence interval of 2.8. In the Nordic study, which included the Norwegian AT relatives, there was a 2.9-fold increase in the risk for breast cancer among all women under the age of 55 and a risk close to that of the general female population for women in the age group 55 years or more. In females verified *ATM* heterozygous the increased risk of breast cancer was 2.7-fold in all age groups, and 5.2-fold in females below the age of 55. Thus, the risk of breast cancer in all verified *ATM* heterozygote females was lower in the Nordic study than the lower limit of the 95% confidence interval found in the Norwegian study, suggesting that different mutations infer a different cancer risk. The Norwegian mutations are only found in two Nordic alleles outside of Norway.

In the Nordic study, the data did not convincingly point to a trend of increasing risk of breast cancer with each increment in the probability of being an *ATM* mutation carrier. The derived risk estimates on the potential role of *ATM* heterozygosity were to a large extent driven by the highly increased risk for breast cancer seen in mothers of probands. Other large studies of blood relatives of patients with AT (Janin *et al*, 1999; Inskip *et al,* 1999; Swift *et al*, 1991; Athma *et al*, 1996) have consistently shown an increased incidence or mortality (UK study) of breast cancer among female family members. The combined data from the published studies of breast cancer risk in female relatives of AT patients demonstrate a substantial and consistent increase in the risk for mothers. The existing international data on the risks for breast cancer of other female relatives are, however, still not conclusive, and convincing data to support a simple relationship between likelihood of ATM heterozygosity and risk of breast cancer has not yet been presented (see discussion in Paper II). In the Norwegian study, SIR of breast cancer in verified *ATM* heterozygote females other than mothers of probands was significantly 2.2-fold increased. However, this estimate is only based on the observation of 18 heterozygote individuals. The discrepancy between the Norwegian and the Nordic study can be a result of inaccurate carrier estimates caused by a greater degree of consanguinity than anticipated in the Nordic study or that different mutations infer different cancer risks since Norwegian *ATM* alleles only recur in two alleles in another Nordic country. A recent study has suggested that truncating mutations in different domains of the ATM protein infer different cancer risks (Cavaciuti *et al*., 2005).

An alternative hypothesis of a confined risk of breast cancer restricted to mothers of AT children, as presented in the absence of a gradient of breast cancer incidence by increasing probability of being a gene carrier, is that giving birth to an AT child or having a pregnancy with a foetus affected with AT affects the mother's breast cancer risk - in combination with or regardless of any effect of her *ATM* heterozygosity. Microchimerism during pregnancy, i.e. the phenomenon that foetal cells may pass into the maternal circulation and tissues, might play a role in the highly increased risk of breast cancer seen among the mothers giving birth to an AT child. The presence of foetal AT cells in the circulation or tissues of the mother may contribute to the development of maternal breast cancer (see discussion Paper II).

Risk of cancers other than female breast

The Nordic study found a slight increased risk for cancers at all sites excluding breast, which reached statistical significance for female relatives only. The Norwegian study, however, found significantly elevated SIRs of cancer at all sites for male, female and both sexes combined in the verified *ATM* heterozygote. No cancers were calculated independently in the Norwegian study other than breast, since the numbers were low. In the Nordic study there was a tendency for slight but non-significant elevations in risk for most cancer types. Especially, female relatives had excess numbers of cancers of the gall bladder and liver, which correlated with the mutation carrier probability of the subjects, but which was not replicated among male relatives. The slight increase seen for ovarian cancer was not correlated with mutation carrier probability or familial proximity to the proband; this was also the case for the increased risk for malignant melanoma in male relatives. A study done in the US has (Swift *et al,* 1991) reported a significant 2.5-fold increase in the risk for cancers at all sites combined in male relatives (73 observations) compared with that of spouses of female relatives (19 observations), and a significant 3.9-fold increase in all cancers in male obligate heterozygote (18 observations). These findings concur with the findings in the Norwegian study: a significant 2.1-fold increased risk for cancer at all sites in males with an upper limit of the confidence interval of 3.9. A risk estimate for all cancers other than breast in female relatives was not made in the US study, but re-calculation on the basis of the data reported in the paper indicates that the risk was lower than that of male relatives. The Norwegian study support the findings that the risk of cancer in females other than breast (1.7 fold increased) is lower than the risk of cancer at all sites for males. In the Nordic study, there is no support for a substantially increased risk for cancers among male relatives. In a separate analysis of the incidence of cancers at sites other than the breast, the French study obtained an overall RR of 0.9 for both sexes combined on the basis of 93 observations (Geoffroy-Perez *et al,* 2001). The differences reported between the different populations may be due to the type of *ATM* mutations occurring in these populations.

Furthermore, in the Norwegian study, the individuals heterozygous for the Rendal-mutation and those with other types of mutations, had significantly elevated risks for males, females and both sexes combined of cancer at all sites for both groups. For both the carriers of the Rendal-mutation and carriers of other mutations, the highest risk was for breast cancer in

females. The risks of cancer at all sites and breast cancer in females, was non-significantly different in carriers of the Rendal-mutation than in carriers of other mutations. Thus, from the Norwegian study we cannot conclude that the Norwegian founder infers a different cancer risk than the other mutations found in the Norwegian population without further studies on larger cohorts.

The main limitation of the Nordic and Norwegian studies, as well as other studies of cancer in AT families, is the small number of individuals included, and with associated low precision in risk estimation for site-specific cancers, including female breast cancer, ovarian cancer, lymphomas and leukaemias. There is a need for increased international collaboration in the study of cancer in AT families and, if feasible, a combined analysis of the available study materials. The hypothesis of breast cancer risk related to being pregnant with an AT-affected child also needs to be pursued.

The Pro/Arg polymorphism in mutated *TP53*

In our study there was an observed skewed occurrence of somatic *TP53* mutations on the Arg72 allele in breast carcinomas suggesting that this combination gives breast epithelial cells a growth advantage, which may increase the risk of malignant transformation and development of cancer. However, in contrast to the breast cancer cases, no difference in the frequency of mutations between the Pro/Pro and Arg/Arg homozygote was found in colorectal cancer. The spectrum of *TP53* mutations is different between these two tumour types[k], partly attributable to tissue-specific differences in carcinogen exposure and in metabolism (reviewed in Hainaut *et al*., 2000). The effect of coexistence of the Arg72 with a mutation may modify the TP53 protein structure in a way that interferes either with the protein's ability to achieve sequence specific binding to DNA or with the interaction and recruitment of the transcription machinery, causing an altered transcription pattern (Campomenosi *et al*., 2001). Another possibility is that the Arg72 may modify the mutant TP53 protein's ability to bind to and interact with other proteins such as, for example, TP73, (Di Como *et al*., 1999; Marin *et al*., 2000), which may interfere with TP73-induced apoptosis. The same level of skewed

[k] The TP53 mutation database: www.iarc.fr/P53/index.html. Accessed 21. January 2005

distribution of mutations residing on the Arg72allele as seen for all type of mutations was also seen for missense mutations, giving no evidence for a stronger effect of such mutations. However, missense mutations are of many different types, and classifications according to structure or function in different cell types in larger series may give other results. The more severe changes like deletion, insertion, nonsense, and splice mutations may lead to a truncated protein or lack of protein where a codon 72 polymorphism has no modifying impact. Analyzing non-missense mutations as one group with respect to the codon 72 homozygotes gave no skewed distribution ($P = 0.710$). The number was, however small, and even truncated TP53 proteins may have an impact through mechanisms like inactivating other proteins (*e.g.,* TP73) if their interacting domain is intact and the protein is stable. It cannot be excluded that the two polymorphic variants may have different effects also on such mutants.

The effects of the Arg 72allele on mutant TP53, were associated with breast but not colon cancer development. A plausible explanation could be that the TP53 mutants in colon cancer are of a different nature than in breast cancers. TP53 mutants in colon cancer have been predicted to have a predominantly dominant negative effect on wildtype TP53, and it has been proposed that it is only the recessive mutations that are preferentially located on the Arg72 allele. The dominant negative mutants have been suggested to be independent of the codon 72 polymorphism (Tada *et al*., 2001). Tada *et al.* proposed criteria for classifying the mutations and when these criteria were applied to our series, the frequency of dominant negative mutants were higher in the colorectal cancer cases (83.3%, 30 of 36) than in the breast cancer cases (61.1%, 22 of 36; $P = 0.064$). Although only a minor fraction of the mutants (72 of 175) could be classified according to these categories, these results nevertheless support the hypothesis that a tumourigenic effect of the Arg72 allele only occurs when combined with a somatic mutation of the type seen in breast carcinomas. Additional studies, including functional assays, are warranted to explore the effects of the different combined variants and their role in tumourigenesis in different tissues.

FUTURE PERSPECTIVES

In the *ATM* heterozygotes, there is a need for further studies, in larger cohorts, to reveal the type of mutation and/or the location of the mutation that infers the greater cancer risk. Furthermore, analyses that enlighten the effect of specific mutations in *ATM* on cell and patient phenotype are needed. This can be used diagnostically for AT patients and their families.

Analysing tumours, by whole genome approaches like expression profiling using microarrays, from carriers of *ATM* mutations will contribute to the understanding of the *ATM* gene in breast carcinogenesis. Further studies with emphasis on the *TP53* gene are needed to elucidate whether there is an association with *TP53* mutations in the tumour tissue and *ATM* heterozygosity and whether the Arg72 allele, found to be associated with *TP53* mutations in breast cancer, plays a role in breast cancer carcinogenesis in *ATM* heterozygotes. Also, tumours from mothers of AT probands need to be analysed, to contribute to our understanding of the particular high risk of breast cancer in these mothers.

Since AT is a rare disease, international collaboration is needed to be able to collect large enough cohorts of verified carriers to perform such studies.

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Pedigree Analyses, *ATM* Mutations and Cancer Risk in Norwegian Families with Ataxia Telangiectasia

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Abstract

Purpose: **Epidemiological studies have consistently shown elevated rates of cancer, especially of the female breast, in blood relatives of patients with the rare autosomal, recessive disease, ataxia telangiectasia (AT). A large proportion of the members of AT families are carriers of AT-causing gene mutations found in the *ATM* gene. We here report pedigrees, molecular analysis and cancer incidence in 18 Norwegian AT-families including 20 probands and 168 blood relatives.**

Material and methods: Blood relatives of patients with verified AT were identified through population registry linkages, and the occurrence of cancer was determined from the cancer registry files in Norway and compared with national incidence rates. DNA was isolated from blood or tumour tissue of AT children and their relatives. DNA from the AT patients or their parents was screened for ATM germ-line mutations by protein truncation test and/or heteroduplex analysis using DHPLC followed by sequencing to determine the nature of the mutation. DNA from the more distant relatives was analysed for the respective mutation segregating in that branch of the family.

Results: Seven cancers occurred amond the AT probands, 0,06 expected, yielding a standardized incidence ratio (SIR) of 119 (95% confidence interval (CI), 88-181). Of the 168 blood relatives tested, 85 were found heterozygous for *ATM* mutations. 23 cancers were found among these individuals, (SIR 2.2; 95%CI 1.7-3.0). Invasive breast cancer was found in 6 cases (SIR 3.6; 95%CI 2.8-5.0), of whom 3 where mothers of probands. One Norwegian founder mutation accounted for 47% of disease causing alleles and there is a high degree of consanguinity in the Norwegian AT population. Carriers of the founder mutation separated from carriers of other mutations had an elevated cancer risk for all sites, and the highest risk was for breast cancer in females. Considering carriers for other mutations, a slightly higher cancer risk was seen in these individuals, other than female cancer at all sites, although this was not statistically significant. When DNA from both blood and tumour were analysed for the same individuals, some discrepancies were detected and possibly attributed to the genetic instability in many tumours.

Conclusion: This study has confirmed that an individual heterozygote for *ATM* mutation has an increased risk of cancer at all sites and females particularly of the breast. No conclusive findings were made suggesting that the Norwegian founder infers

a different cancer risk than other mutations. Epidemiological studies need to be supported not only by accurate pedigrees of the families, but by molecular analysis as well, especially when there is consanguinity. In these families, statistically calculated carrier risk would be inaccurate without this information. Furthermore, blood samples are the reliable source of genetic information and are needed for accurate analysis of carrier status of the *ATM* gene.

Introduction

The rare autosomal recessive disease ataxia telangiectasia is caused by mutations in the *ATM* gene. Mutations leading to the classical AT phenotype, with progressive cerebellar ataxia, telangiectasia of the eyes, immunodeficiency, elevated alpha-feto protein levels, chromosomal instability and a susceptibility to develop leukemia and cancers of lymphoid origin are to a large degree truncating mutations. (Gatti *et al*., 1991) Other mutation types in the ATM gene such as missense mutations have also been found, leading to an AT like disease in the homozygous state or in compound heterozygous for a mild and a severe mutation, usually to a milder phenotype (Gilad *et al*., 1998). The estimated carrier frequencies in the general population is in the order of 0.5–1% for an AT-causing mutation. A heterozygous individual shows no symptoms of AT, however several studies have reported that relatives of AT patients are at three- to fivefold increased risk of developing breast cancer (Swift *et al*., 1991; Geoffrey-Perez *et al*., 2001; Olsen *et al*., 2001) and perhaps other cancers. Molecular studies of breast cancer patients have failed to detect an increased frequency of potentially AT-causing mutations in breast cancer (Vorechovsky *et al*., 1996a Vorechovsky *et al*., 1996b; Chen *et al*., 1998) However, it has been hypothesized that "milder" mutations leading to conservative amino acid changes, inhabit a greater cancer risk in the heterozygote state than truncating mutations. This is due to a possible dominant negative effect of mutated ATM on the wildtype ATM when the mutated ATM is not truncated (Scott *et al*., 2002). Truncated protein is most commonly rapidly degraded by proteases. When this does not occur, as in the case of many missense mutations, mutated ATM will form dimers or multimers with wildtype ATM inhibiting the functional properties of the wildtype protein (Chenevix-Trench *et al*., 2002). No significant correlation between mutation type and increased cancer incidence has been found so far in the AT families. However, it has been suggested that truncating mutations in domains particularly involving TP53 binding, lead to an increased cancer risk

(Cavaciuti *et al*., 2005). Thus, the question is if both the type and location of mutation in the ATM gene might have a significant impact upon cancer risk.

A Norwegian founder mutation in exon 24 (Laake *et al.*, 1998), causing 12 amino acid substitutions and truncation of the protein, has previously been reported to be responsible for 55% of the Norwegian disease causing alleles (Laake *et al*., 1998). Epidemiological studies in the Nordic countries have previously reported an increased cancer risk for heterozygotes in this population (Olsen *et al*., 2000 and Olsen *et al*., submitted). However, there is a high degree of consanguinity in the Norwegian population, which makes statistical evaluation of carrier status inaccurate. Also, because of the high incidence of the Norwegian founder mutation it is possible to statistically estimate whether individuals with truncating mutations in this specific location have an increased cancer risk compared to other mutations found in Norwegian AT families. Here, we present pedigrees of the Norwegian AT families, their *ATM* mutation carrier status based on molecular analysis and calculated cancer risks for *ATM* heterozygous individuals.

Materials and methods

A total of 18 families of Norwegian origin were studied. All diagnoses were made on the basis of internationally recognized clinical and laboratory criteria obtained from the medical records of the patients. Absolute criterion for inclusion was progressive cerebellar ataxia. Supporting criteria were telangiectasias, ocular apraxia, dysarthria, history of infections (minimum one episode of pneumonia), elevated alpha-feto-protein, chromosome rearrangements involving chromosomes 7 and 14, and/or increased chromosome breakage and decreased IgA or IgG_2. Informed consents were gathered through the Norwegian study coordinator and the immediate family of probands. One family chose not to participate (NOAT12). Blood samples, lymphoblastoid cell lines, fibroblasts and tumour tissue from AT probands and their relatives were collected from all cases verified or suspected AT and the families were included as the diagnoses were verified. One family was excluded on this basis. (NOAT19). All of the cancers were confirmed by the Norwegian cancer registry and/or by other pathologists. The malignant neoplasms identified in the cohort of relatives were classified according to the International Classification of Diseases, 7th Revision. The registration and coding practices of the cancer registry has been described elsewhere (Tulinius et al, 1992).

Mutation analysis

Several mutation analysis techniques have been used to locate the disease causing ATM mutation in AT probands as the Norwegian AT study has been ongoing for over a decade. DGGE (denaturing gel gradient electrophoresis, described in Børresen *et al*., 1998) has a high sensitivity for detecting the Rendal-mutation, however, sensitivity for other mutations than the Rendal is low (Laake *et al*., 1998). With the PTT (Protein Truncation Test, reviewed in Den Dunnen and Van Ommen, 1999) we detected other mutations that cause premature termination of translation or large in frame deletions (Telatar *et al*., 1998 and Laake *et al*., 2000). Heteroduplex analysis by DHPLC (denaturing high pressure liquid chromatography) has proven the most sensitive and mutations are found where the older techniques failed to detect mutations (conditions described in Bernstein *et al*., 2003 and method reviewed in Xiao and Oefner, 2001) Thus, screening for ATM germline mutations has been done on DNA from the AT patients or their parents by protein truncation test and/or heteroduplex analysis using DHPLC. When a variant pattern was observed by DGGE, PTT or DHPLC the DNA or cDNA, was sequenced in both directions using DyeDeoxy Terminator Cycle sequencing kits (Applied Biosystems, Inc., Foster City, CA) using an ABI 3100 DNA sequencer (Applied Biosystems, Inc).

When the probands mutations were identified, their blood relatives were analysed with heteroduplex analysis or direct sequencing on DNA extracted from blood samples or paraffin embedded tumour tissue (protocol described in appendix).

Statistical analysis

The standardized incidence ratio (SIR) of cancer was calculated for carriers of an *ATM* mutation. National incidence rates for the tumour categories were calculated according to sex, age (in 5-year groups), and 5-year calendar periods and applied to the person-years of observation in the respective national subcohorts to obtain the number of cancers expected. The observed and expected numbers of cancers in the individuals confirmed heterozygote were used to calculate SIRs. The 95% confidence intervals for the SIRs were calculated by assuming a Poisson distribution of the observed cancers. Statistical calculations were made using SPSS, version 11.5, and Excel 2000.

Results

Screening for the ATM mutations in the AT probands

A total of 20 AT patients in 18 Norwegian families were included in the study. Nine probands were alive as of 311202, and the oldest Norwegian proband reported, died at age 33. The patients were born in the period 1962-2002. The diagnosis of the proband is supported by identification of a disease causing mutation in the ATM gene in both alleles in 17 families. In one family only one disease causing allele has been found. Previous linkage studies have concluded that the other mutation is a de novo mutation (Laake *et al*., 1998), however it has yet to be found (the patient has died and there is no more DNA). Table 1 shows the distributions of mutations and the expected consequences for the protein. 12 unique disease-causing mutations are found in the Norwegian families (of which three are not previously published) Three**,** previously reported mutations were found in new families. The mutations found in the Norwegian AT families have different consequences for the ATM protein: 74 % (26/35) of the parental branches have frameshift mutations that subsequently lead to truncation of the protein, 14 % (5/35) have splice site mutations that lead to skipping of exons, 6 % (2/35) have nonsense mutations, inducing stop codons at the mutation site, 3% (1/35) have double substitutions and 3 % (1/35) have missense mutations. With this low fraction of mutations with "mild" consequences for the protein, we chose not to make statistical calculations of SIR segregating for different type of mutations.

Pedigree analyses and mutation status detection

A previously identified founder mutation in exon 24 (Laake *et al*., 1998) which changes the sequence from ATC to TGAT inducing a stop codon thirteen amino acids downstream, was found in 11 out of the 18 families, in 18 out of 39 found proband alleles (46%) Seven patients were homozygotes for this so-called "Rendal-mutation" and four patients were compound heterozygous. Geneological studies have so far identified a common ancestor for 5 of the Norwegian AT families with the founder mutation (28 %). The common ancestor was born in approximately 1495. Figure 1 shows how the families are connected. Further genealogical studies would most likely reveal that even more Norwegian AT families are of common descent since the allele is so frequent in the Norwegian AT probands.

In addition to the large pedigree for the Norwegian founder mutation, pedigrees were made of all the Norwegian AT families. The pedigrees in figure 2 contain ATM carrier status for those

tested, cancer diagnosis and age at time of diagnosis. The pedigrees were made in order to trace the disease causing mutation and discover consanguinity. The presence of consanguinity found in the Norwegian population affects carrier probabilities, and thus, carrier probabilities will be inaccurate without knowing the extended pedigree and the intermarriages that have occurred over time.
The father in family NOAT 7 and mother in NOAT 8 carry the same mutation and haplotype (Laake *et al*., 1998). They were born in the same community. The mother in NOAT 7 carries the same mutation found in both parents in family NOAT 13 where the parents probably have common ancestors. The maternal and paternal grandfathers carry the same mutation. In two families, two siblings were affected. (Laake *et al*., 2000)

A total of five mutations recur in the Norwegian families. Six of the mutations are also found outside of Norway, 2 in Denmark (1 allele of each), 3 in the US (1 allele of each), 1 in the Netherlands (3 alleles) and one in Great Britain (1 allele) (Telatar *et al*., 1998; Laake *et al*., 1998; Li and Swift, 2000, van Belzen *et al*., 1998; Stankovic *et al*., 1998; Teraoka *et al*., 1999 and Laake *et al*., 2000).

Gene testing for the family mutation was conducted for a total of 168 blood relatives of AT probands from all 18 families. DNA from these relatives was analysed for the respective mutation segregating in their branch of the family (table 2). 140 analyses were done on the basis of a blood sample from a live relative and 28 solely on the basis of tissue from an archival tumour block. When possible, DNA from both normal and tumour tissue was extracted from the paraffin block. For 13 individuals both blood and tissue samples were available for analysis. In three of the heterozygote cases, the results from the analysis of DNA from the tumour block did not correspond with blood result. Only the wildtype allele was found, indicating a loss of the mutated allele.

Cancer risk

A total of 7 cancers occurred among the AT probands and 23 cancers in the ATM heterozygous. One AT proband and four heterozygous individuals had two primary cancers originating at different sites. Thus, in the probands, 7 cancers were observed with 0,06 expected, yielding a SIR of cancer at all sites of 119 for both sexes with 95% confidence interval of 88-91 (table 3). It was lower for males, with a 45-fold increased risk of cancer (33-

73) and higher for females, 163-fold increased risk (105-359). The cancers were lymphomas and leukemias, except for one liver cancer.

In the mutation verified heterozygous individuals 23 cancers were observed, with 10,7 expected, yielding a 2.2-fold increased risk of cancer at all sites (95%CI 1.7-3.0) (table 4). In females 6 breast cancers were observed and 1,7 expected, SIR 3.6 (2.8-5.0). Three of the breast cancers were in mothers of a proband. If the mothers were excluded, risk of breast cancer for females was 2.2 (1.6-3.0). For female cancer other than breast the SIR for all females was 1.7 (1.2-2.6).

When separating the heterozygous into two groups, one with the founder mutation and the other group with mutations different than the founder, observed cancers in both sexes with the founder mutation were 13 and expected 6,3, SIR 2.1 (1.5-3.4). Females with the founder had an SIR of breast cancer of 3.0 (2.2-5.0). In the group with mutations different from the founder, in both sexes 10 cancers were observed, with 4,4 expected, SIR 2.3 (1.7-3.5). Females with other mutations than the founder had a SIR of breast cancer of 4.3 (3.0-8.1).

Discussion

In families with a high degree of consanguinity and especially in the presence of a founder mutation, it is essential that epidemiological studies are combined with molecular studies and pedigree analyses. Five of the families carrying the founder mutations have been linked together, but most likely there are even more families attached to the "Rendal-pedigree". Thus, any estimation of carrier probability – especially background probability - would be inaccurate in several of the Norwegian AT families, since they have a much greater risk of being carriers than the rest of the population due to consanguinity. In other words, without molecular analysis it is impossible to reveal a true correlation between carrier status and increased cancer risk.

The *ATM* heterozygous have significantly elevated SIRs of cancer at all sites for male, female and both sexes combined. The risk of female breast cancer is the most elevated cancer type, significantly higher than other female cancers, which is also elevated. When we divide the heterozygous into two groups, those carrying the Rendal-mutation and those with other types

of mutations, we find significantly elevated risks for males, females and both sexes combined of cancer at all sites for both groups. For both the carriers of the Rendal-mutation and carriers of other mutations, the highest risk was for breast cancer in females. The risks of cancer at all sites and breast cancer in females, was non-significantly different in carriers of the Rendal-mutation than in carriers of other mutations. Thus, we cannot conclude that the Norwegian founder infers a different cancer risk than the other mutations found in the Norwegian population without further studies on larger cohorts.

The risk of female breast cancer was 3.6-fold increased in the Norwegian study in all female relatives verified *ATM* heterozygous with a lower limit of the 95% confidence interval of 2.8. In a Nordic study (Olsen, submitted), which includes the Norwegian AT relatives, there was a 2.9-fold increase in the risk for breast cancer among all women under the age of 55 and a risk close to that of the general female population for women in the age group 55 years or more. In females verified *ATM* heterozygous the increased risk of breast cancer was 2.7-fold in all age groups, and 5.2-fold in females below the age of 55. Thus, the risk of breast cancer in all verified *ATM* heterozygote females was lower in the Nordic study than the lower limit of the 95% confidence interval found in the Norwegian study, suggesting that different mutations infer a different cancer risk as suggested by others (Caviacuti *et al*., 2005). The Norwegian mutations are only found in two Nordic alleles outside of Norway.

When tumour samples are collected, more heterozygous individuals with tumours will be detected than heterozygous without tumours. As well, individuals with cancer might be more interested in and available for giving blood samples than healthy individuals. This is a source of selection bias in all studies were complete ascertainment is not possible.

The discrepancy seen between the results from blood DNA and tumour DNA from the same individual is possibly due to a loss of heterozygosity in tumour tissue. If one allele is lost this would not give heteroduplex formation during PCR and the sample will be interpreted as a wildtype.

More than one block was available from tumours for several individuals. In three individuals, the analysis done on different blocks gave different results. Both normal tissue and tumour tissue from the blocks were analysed, and the results differed for both normal and tumour

tissue. These individuals were excluded from the study, as we had no conclusive mutation status data. To explain these findings we attribute this to both tumour hetereogeneity and the early occurrence of LOH in some cancers, which might affect adjacent, hyperplastic tissue (Chin *et al*., 2004). The *ATM* gene does not seem to follow the classical Knudson two hit modell for tumour suppressors. The mutated *ATM* allele, not the healthy allele, is lost in some of these tumours due to genetic instability. In mutation analysis based on heteroduplex formation, blood samples are the only reliable source of information on carrier status and blood samples should be taken from everybody.

Conclusion

This study has confirmed that an individual heterozygote for *ATM* mutation has an increased risk of cancer at all sites and females particularly of the breast. No conclusive findings were made suggesting that the Norwegian founder confers a different cancer risk than other mutations. Epidemiological studies need to be supported not only by accurate pedigrees of the families, but by molecular analysis as well, when there is consanguinity. In these families, statistically calculated carrier risk is inaccurate without this information. To further investigate whether different mutations actually infer different cancer risks and to what degree, mutation analysis of all participants and pooling of international cohorts are needed. Furthermore, blood samples are the reliable source of genetic information and are needed for accurate analysis of carrier status of the *ATM* gene.

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Breast and other cancers in 1445 blood relatives of 75 Nordic patients with ataxia telangiectasia

Running title: Cancer in AT families

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ABSTRACT

Epidemiological studies have consistently shown elevated rates of breast cancer among female blood relatives of patients with ataxia telangiectasia (AT), a rare autosomal recessive disease. A large proportion of the members of AT families are carriers of AT-causing gene mutations in ATM (Ataxia Telangiectasia Mutated), and it has been hypothesized that these otherwise healthy carriers are predisposed to breast cancer. Blood relatives of patients with verified AT in the Nordic countries were identified through population registry linkages, and the occurrence of cancer was determined from cancer registry files in each country and compared with national incidence rates. The ATM mutation carrier probabilities of relatives were assigned from the combined information on location in family, consanguinity, if any, and supplementary carrier screening in some families. Among the 1445 blood relatives of AT patients, 225 cancers were observed, with 170.4 expected, yielding a standardized incidence ratio (SIR) of 1.3 (95% confidence interval (CI), 1.1–1.4). Invasive breast cancer occurred in 34 female relatives (SIR, 1.7; 95% CI, 1.2–2.4) and was diagnosed in 21 women before the age of 55 (SIR, 2.9; 95% CI, 1.8–4.5) including seven mothers of probands (SIR, 8.1; 95% CI, 3.3–17). When the group of mothers was excluded, no clear relationship was observed between the allocated mutation carrier probability of each family member and the extent of breast cancer risk. We concluded that the increased risk for female breast cancer seen in 66 Nordic AT families appeared to be restricted to women under the age of 55 and was due mainly to a very high risk in the group of mothers. The findings of breast cancer risk in mothers, but not other likely mutation carriers, in this and other studies raises questions about the hypothesis of a simple causal relationship with ATM heterozygosity.

Key words: ATM heterozygosity; early-onset breast cancer; cancer predisposition; familial cancer

INTRODUCTION

While mutations of both alleles of the ATM (Ataxia Telangiectasia Mutated) gene cause the rare autosomal recessive disorder ataxia telangiectasia (AT), heterozygous carriers of an ATM allele are healthy. Several studies, however, have estimated carriers to be at three- to fivefold increased risk for developing breast cancer (Swift *et al,* 1991; Athma *et al,* 1996; Inskip *et al*, 1999; Janin *et al,* 1999; Geoffroy-Perez *et al,* 2001; Olsen *et al,* 2001) and perhaps other cancers. With calculated mutation carrier frequencies in the general population in the order of 0.5–1% for ATM gene mutations, ATM heterozygosity might be responsible for a sizeable proportion of breast cancers in the (female) population (Easton, 1994).

We previously published a cohort study of cancer incidence in 1218 blood relatives of 56 Nordic AT patients from 50 families (Olsen *et al,* 2001). In order to increase the statistical power of the study, we added 16 more families, and 227 relatives. We extended the follow-up for cancer incidence and a further correction of the mutation carrier probabilities based on supplementary mutation carrier testing of relevant members in some of the families. The results of this updated study are reported here.

MATERIALS AND METHODS

In each of the participating countries (Denmark, Finland; Norway and Sweden), paediatric neurologists, paediatric immunologists, medical geneticists, cytogenetic laboratories and institutions for disabled children were requested to report cases of verified or suspected AT (from 1950 through 2002) to the country's study coordinator. The medical records were reviewed with respect to absolute and supporting criteria for the clinical diagnosis AT, as previously described (Olsen *et al,* 2001). Blood samples, lymphoblastoid cell lines or fibroblasts were available for most families,

either from the proband (or affected siblings) when alive or from the parents. The ATM gene was screened for disease-causing mutations by heteroduplex analysis using DHPLC or protein truncating testing with subsequent sequence analysis at the cDNA and genomic level to identify the nature of the mutation (Laake *et al*, 2000; Bernstein *et al*, 2003). When biological samples were not available, the diagnosis of AT was based on the clinical and laboratory criteria.

Tracing of relatives for construction of pedigrees was based on data from the computerized national civil registration systems of the Nordic countries, as these systems make use of the personal identification number (PIN), unique for each citizen, allowing accurate linkage of registry information on parents and their offspring. These registration systems were started in 1960 in Norway, 1961 in Sweden, 1967 in Finland and 1968 in Denmark, when the PIN was assigned to all citizens alive at that date; for individuals born after that date, the PIN is assigned at birth. Information on more distant ancestors was derived from manual local population and church registers. Finally, follow-up information on date of death or emigration of blood relatives was obtained from the aforementioned national civil registration systems and from the national mortality files. Additional details are given in the previous publication (Olsen *et al,* 2001).

Data on blood relatives of AT patients were linked to the national cancer registry of the respective Nordic country by the subjects' PIN or, if they had died before the civil registration systems were computerized, their date of birth, date of death and name (Olsen *et al,* 1993). The period of follow-up for the occurrence of cancer among siblings, cousins, uncles, aunts and grandparents' siblings extended from the date of birth or the inception of national cancer registration (Denmark, 1943; Norway, 1953; Finland, 1953; Sweden, 1958), whichever came later, to the date of death or emigration or the end of study (31 December 2000 in Finland and Sweden and 31 December 2002 in Denmark and Norway), whichever came first. Similar rules were applied to the parents, grandparents and great-grandparents of the AT patients, except that follow-up was started at the

earliest from the date of birth of the individual who was in direct line to the proband (e.g. the date of birth of the parent of the proband for grandparents). The malignant neoplasms identified in the cohort of relatives were classified according to the International Classification of Diseases, 7th Revision. The registration and coding practices of the four cancer registries have been described elsewhere (Tulinius *et al,* 1992).

Statistical analysis

The expected numbers of cancers were calculated by multiplying the number of person-years of family members by the national cancer incidence rates for men and women in 5-year age groups and calendar periods of observation. Observed and expected numbers of cancers were pooled among countries, and standardized incidence ratios (SIRs), taken as the ratio of observed-to-expected cancers, were determined. The 95% confidence intervals (CIs) of the SIRs were calculated assuming a Poisson distribution of the observed cancers (Bailar and Federer, 1964).

Cancer risk analyses were also undertaken after stratifying the study population according to their estimated gene carrier probability (1.0, 0.67, 0.5, 0.25, background). The individually assigned and estimated probability was the product of the location of the particular relative in the family pedigree (taking into account any information on consanguinity in the family) and the outcomes of any gene testing performed on members of the family, in addition to that already conducted on the proband, affected siblings and/or parents. For instance, if a grandmother tested positive for the one of the mutations of the proband, this changed the likelihood that the grandmothers' and the grandfathers' ancestors had been mutation carriers. In order to avoid bias due to selective testing of survivors in the families, however, the mutation carrier probability of the tested relatives themselves (which often was conducted years after the entry of the relative into the study cohort) were kept unchanged

in the risk analyses. The number of actual mutation carriers was estimated by multiplying the probability of being a carrier by the number of subjects in each subgroup of female relatives. On this basis, the relative risk for female breast cancer associated with heterozygosity for ATM mutation was estimated assuming that the excess risk for breast cancer observed in the entire group of female relatives can be ascribed to the subgroup of mutation carriers only (Olsen *et al,* 2001).

RESULTS

A total of 75 AT patients from 66 families (24 patients from 21 families in Denmark, six patients from six families in Finland, 21 patients from 19 families in Norway and 24 patients from 20 families in Sweden) were included in the study. In nine families, two siblings were affected. The patients were born in the period 1949–2002 and all had a diagnosis of AT on the basis of clinical and laboratory findings. Biological material was available from the proband, an affected sibling and/or the parent(s) in 54 of the families representing 62 AT patients. A disease-causing mutation of the ATM gene was identified in both alleles in 57 patients from 50 of these families, while in five patients from four families material was available from only one parent, and therefore only one mutation was identified. In the remaining 13 patients from 12 families, biological material was not available, and the diagnosis was based entirely on clinical and laboratory information from the medical records. In two patients (from different families) who had both their mutations identified, the mutation of the maternal allele was shown to be *de novo*. Consequently, in the risk analyses stratified by carrier status these mothers and their ancestors were regarded carriers of a wild type AT allele. The pedigrees indicated consanguinity in eight (12%) of the 66 families (mainly cousin–cousin marriages); however, homozygosity in the proband for a specific mutation was seen in 19 (38%) of the 50 families for which full allelic information was available. Of these, seven were a

specific Norwegian founder mutation, the so-called Rendal mutation, located in exon 24 (3245delATCinsTGAT) (Laake *et al*, 2000).

In addition to gene testing of affected siblings and parents of probands, testing was conducted for 37 relatives from 13 Norwegian families: 21 on the basis of a blood sample from a live relative and 16 on the basis of tissue from a paraffin embedded tumour block from a deceased relative. This increased the number of obligate carriers (from 144 to 152) as well as the numbers of relatives with gene carrier probabilities of 0.5 (from 441 to 459) and 'background' (from 42 to 136). On the contrary, it reduced the numbers of relatives with gene carrier probabilities of 0.67 (from 75 to 73) and 0.25 (from 873 to 755).

Of the 1575 unaffected blood relatives successfully identified, 130 had died before the date of eligibility, leaving 1445 relatives for cancer risk analysis. These consisted of 733 men and 712 women; 128 parents, 84 siblings, 189 grandparents, 241 uncles and aunts, 400 cousins, 170 great-grandparents and 233 grandparents' siblings. The entire group represented some 46 000 person-years of follow-up (mean, 31.7 years; range, 0–60 years), during which time 225 cancers were observed (106 in men and 119 in women), with 170.41 expected, yielding statistically significant SIRs of 1.28 (95% CI, 1.1–1.4) overall, 1.15 (0.9–1.4) for men and 1.42 (1.2–1.7) for women.

Of the 119 cancers in women, 34 were of the breast (all unilateral), with 19.51 expected, yielding a statistically significant SIR of 1.7 (Table 1). The association with breast cancer was particularly strong in the group of 64 mothers (the mother was missing in each of two Swedish families), who *a priori* were assumed to be ATM mutation carriers, although genetic testing revealed that two was not (SIR of 6.7 and a lower limit of the 95% CI of 2.9). In the remaining, combined group of female relatives, the SIR for breast cancer was a modest 1.4 and non-significantly increased, although with an indication of an excess risk for grandmothers, grandmothers' sisters and great-grandmothers. Table 1 also shows the risk for breast cancer according to estimated mutation carrier probability.

Among the ten women (mostly grandmothers) deemed to be mutation carriers by virtue of pedigree position or genetic testing, in addition to the 62 mothers being obligate carriers, there were no cases of breast cancers, decreasing the overall SIR of obligate carriers to 4.4, which still represents a significant risk elevation. The combined group of likely mutation carriers (probabilities of 0.67, 0.50 and 0.25) had a marginally significant, modest 60% increase in the risk for breast cancer, but no increase in risk with higher likelihood of being a mutation carrier (Table 1). On the basis of the carrier probability distribution given in the lower part of Table 1, 287 of the 712 female relatives included in the analysis were estimated to be ATM mutation carriers, i.e. 40%. Assuming the existence of a true link between a mutated ATM allele and breast cancer, our data indicate that ATM heterozygosity on average infers a 2.9-fold (95% CI, 1.9-4.4) increase in the risk for female breast cancer.

Of the 34 cases of breast cancers among women, 21 were diagnosed before the age of 55 years (SIR, 2.9) and 13 at the age of 55 or older (SIR, 1.1), suggesting the occurrence of early-onset breast cancer in these families (Table 2). Again, the association was clearly strongest for the mothers, with an 8.1-fold increase in risk in the age range below 55 years and a 3.3-fold increase as the lower 95% CI. Significantly increased risks were also seen for grandmothers, grandmothers' sisters and great-grandmothers when data were available for that age range. Nevertheless, detailed data on risk elevation by mutation carrier probability did not reflect an increasing risk with higher likelihood of being a carrier (Table 2).

No cases of breast cancer were observed among male relatives (0.1 expected).

When we analysed the risk for breast cancer by selected characteristics of the probands and the families, we did not observe any tendency to higher risks for breast cancer among female relatives, including mothers, in the eight reported consanguineous families (SIR, 1.8), in the 19 families with probands homozygous for a mutation (SIR, 1.3), including the seven with Rendal mutation (1.5), or

in the nine families of probands with cancer (1.8), than we did among female relatives of the remaining groups of families, yielding SIRs of 1.8, 1.9, 1.8, and 1.7, respectively. Among the nine mothers with two affected offspring, one case of breast cancer was observed (SIR, 4.3), and among the remaining mothers with one affected child, seven cases were observed (SIR, 7.2).

A total of 191 cancers were observed at sites other than the breast (106 in men and 85 in women) in the combined group of relatives, yielding a SIR of 1.2 (Table 3). The slightly but significantly increased overall risk was significant in women (SIR, 1.3) but not in men (1.2). As seen from the table, there was a tendency for increased risks for cancers at most sites, but malignant melanoma of the skin was the only site for which the increase reached statistical significance. The excess was seen primarily in men, but with no correlation to their likelihood of being mutation carriers; three of the 10 cases were seen in the subgroup with 'background' mutation carrier probability, when 0.6 was expected.

Because of the known genetic link between cancers of the breast and ovary, we reviewed family details for the eight AT family members with cancer of the ovary (SIR, 1.7). Two cases occurred in aunts, three in grandmothers and three in grandmothers' sisters, but no case occurred in mothers. There was no indication of an increased risk with higher likelihood of being a mutation carrier. Of eight cases of cancers of the liver and biliary passages, six were observed in women, yielding a significantly increased SIR of 3.9; of these, three were in the mutation carrier probability group 1 (0.08 expected; SIR, 36; 95% CI, 7.3–106), none in probability group 0.67/0.5 (0.5 expected), two in probability group 0.25 (0.8 expected), and one in probability group 'background' (0.2 expected). Two cases were seen in men (one in probability group 1 and one in group 0.50) when 0.8 was expected.

In contrast to the findings for breast cancer, the tendency to increased risks for cancers at other sites was not further strengthened when the analysis was restricted to persons aged below 55 years.

DISCUSSION

In this extended and enlarged follow-up study of cancer incidence in 1445 blood relatives of 75 patients with AT, we observed a statistically significant, 2.9-fold increase in the risk for breast cancer among women under the age of 55 and a risk close to that of the general female population for women in the age group 55 years or more. Our observation of an increased risk for early-onset breast cancer, now on the basis of 21 observed cases, corroborates the finding of our initial follow-up study, which was based on 13 cases (Olsen *et al,* 2001). The excess risk for breast cancer was evident in the mothers of the probands but less conspicuous in other female relatives, even those aged below 55. Although ATM heterozygosity in relatives on average was estimated to infer a significant 2.9-fold increased risks for breast cancer, if causal, our data did not convincingly point to a trend of increasing risk with each increment in the probability of being an ATM mutation carrier. These derived risk estimates on the potential role of ATM heterozygosity were to a large extent driven by the highly increased risk for breast cancer seen in mothers of probands. Although the absence of a clear correlation with the likelihood of being a carrier may be due to the small number of breast cancer cases in each probability group, it does appear to detract from the hypothesis of a causal link between the ATM mutation and breast cancer and raises questions about the likelihood of a simple genetic relationship.

The strengths of our study include the cohort design, the identification of study subjects from medical records, the unbiased identification of relatives through population registry linkage, the unbiased ascertainment and validation of cancer through cancer registry linkage, and the long (maximum, 60 years) and nearly complete follow-up of the entire study population. No family in the study was selected due to sporadic ATM gene mutation analyses of tumour tissue from cancer patients in the general population. The gene testing performed on tumour blocks from relatives affected with cancer was done after the identification of the patient and the relatives. Therefore,

only the gene probability score of the ancestors of the tested person changed, and not the score of the person him- or herself. This avoided a bias due to selection of study subjects for testing that by definition had a cancer.

The observed excess risk for breast cancer among mothers is so large that neither chance nor confounding is a feasible explanation. Confounding would be possible if the mothers or other female relatives were less likely than the general population to have children or more likely to have children later in life (Ewertz *et al,* 1990). The reproductive pattern of the 66 families under study did not, however, indicate that either factor is of importance. On the contrary, we may have underestimated the strength of the association in female blood relatives in direct line with the AT patient (mothers, grandmothers and great-grandmothers), because the national rates of breast cancer are influenced by an approximately 30% higher risk for breast cancer among nulliparous women than among parous women. Confounding could also arise if the mothers of children with AT were more likely to undergo screening examinations for the early detection of breast cancer than the general population, because clinicians might be aware of the suggested link between ATM heterozygosity and breast cancer. This suggestion is, however, recent and is not yet widespread knowledge among colleagues or in affected families.

Large studies of blood relatives of patients with AT from France (Janin *et al*, 1999), the United Kingdom (Inskip *et al,* 1999) and the USA (Swift *et al*, 1991; Athma *et al*, 1996) have consistently shown an increased incidence or mortality (UK study) of breast cancer among female family members. In a cross-sectional analysis of 33 cases of breast cancer diagnosed in female relatives of 99 AT families in the USA, in which gene mutations were analysed, Athma and co-workers (1996) found a significantly increased odds ratio of 3.8 for breast cancer among ATM gene carriers compared with non-carriers. This is compatible with our estimate of a 2.7-fold increased risk for breast cancer among female mutation carriers in general. The analysis of the US family data

indicated, however, that the risk was increased among older women (≥ 60 years) in particular, which clearly contrasts with our observation of an increased risk for early-onset breast cancer. Our finding does, however, appear to concur with that of the French study, which based on 29 observed female breast cancer cases in blood relatives of 34 AT families found the excess risk for breast cancer to be higher among female relatives below the age of 45 than among female relatives above that age (Janin *et al,* 1999; Geoffroy-Perez *et al,* 2001). Also, as in our study, the risk for breast cancer among female relatives in the French study seemed to be restricted to the subgroup of presumed obligate carriers (including the mothers of probands), with five observed cancers among young carriers, equivalent to a significantly increased relative risk of 4.6. These findings are compatible with the estimate of 7.1 among young (< 55 years) obligate carriers seen in our study. In the French study, the risks for breast cancer in the subgroups with estimated carrier probabilities of 0,67, 0.50 and 0.25 were similar to that of the general population, also indicating the absence of a clear relationship between mutation carrier probability and breast cancer risk. Unfortunately, the risk for breast cancer among mothers was not given separately. In the study in the United Kingdom, only mothers (obligate carriers) and grandmothers (0.50 probability carriers) were included as female relatives of the 95 AT probands (Inskip *et al,* 1999). On the basis of three observed deaths from breast cancer in each group of female relatives, the risk of mothers was non-significantly increased (SMR, 3.4) and that of grandmothers was close to that of the general population (SMR, 0.9), providing little support to a relationship between risk and carrier likelihood.

Thus, to our minds, the combined data from the published studies of breast cancer risk in female relatives of AT patients demonstrate a substantial and consistent increase in the risk for mothers. The existing international data on the risks for breast cancer of other female relatives are, however, still not conclusive, and convincing data to support a simple relationship between likelihood of ATM heterozygosity and risk of breast cancer has not yet been presented. Our extended data from

the Nordic study showed no indication of variation in the risk for female breast cancer in analyses stratified according to the major characteristics of the probands or the families. An alternative hypothesis for the absence of a gradient of breast cancer incidence by increasing probability of being a gene carrier and the finding of an increased incidence mainly confined to mothers might be that giving birth to an AT child or having a pregnancy with a foetus affected with AT changes the mother's breast cancer risk - in combination with or regardless of any effect of her ATM heterozygosity. One can speculate whether microchimerism during pregnancy, i.e. the phenomenon that foetal cells may pass into the maternal circulation and tissues, play a role in the highly increased risk of breast cancer seen among the mothers giving birth to an AT child. It has been suggested that microchimerism is associated with various immunological conditions of pregnancy and some chronic autoimmune conditions predominantly found in women (Bianchi, 2000), and in one study it has been associated with cervical cancer (Cha *et al,* 2003). It is conceivable that being pregnant with a foetus affected with AT may facilitate this biological phenomenon, and that the presence of foetal AT cells in the circulation or tissues of the mother may contribute to the development of maternal breast cancer. In our study, however, we saw no variation in breast cancer risk after stratification of mothers according to the number (one or two) of offspring affected by AT, although this conclusion is severely weakened by the small number of mothers and outcomes included. Detailed consistent data about pregnancies were not available in this study.

We found a slight increased risk for cancers at all sites except breast, which reached statistical significance for female relatives only. There was a tendency for slight but non-significant elevations in risk for most diagnostic groups. Female relatives had excess numbers of cancers of the gall bladder and liver, which correlated with the mutation carrier probability of the subjects, but which was not replicated among male relatives. The slight increase seen for ovarian cancer was not correlated with mutation carrier probability or familial proximity to the proband; this was also the

case in the increased risk for malignant melanoma in male relatives. In one of their initial analyses of cancer incidence in blood relatives of AT patients Swift et al. (Swift *et al,* 1991) reported a significant 2.5-fold increase in the risk for cancers at all sites combined in male relatives (73 observations) compared with that of spouses of female relatives (19 observations), and a significant 3.9-fold increase in all cancers in male obligate heterozygotes (18 observations). A risk estimate for all cancers other than breast in female relatives was not made in this study, but our re-calculation on the basis of data reported in the paper indicates that the risk was much lower than that of male relatives. There is no good explanation for the observed difference between the two sexes in the US study, and there is no support in our study for a substantially increased risk for cancers among male relatives. In a separate analysis of the incidence of cancers at sites other than the breast, the French study obtained an overall RR of 0.9 for both sexes combined on the basis of 93 observations (Geoffroy-Perez *et al,* 2001). Liver was the only site for which there was a significant increase in risk, on the basis of six observed and 1.5 expected cases; sex-specific risks were, however, not given. Interestingly, there have been a few case-reports of hepatocellular carcinoma in AT patients, all in females (Weinstein *et al,* 1985; Kumar *et al,* 1979).

The main limitation of our study, as well as other studies of cancer in AT families, is small study groups with associated low precision in risk estimation for site-specific cancers, including female breast cancer, ovarian cancer, lymphomas and leukaemias. This indicates the need for increased international collaboration in the study of cancer in AT families and, if feasible, a combined analysis of the available study materials. The hypothesis of breast cancer risk related to being pregnant with an AT-affected child also needs to be pursued.

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Short Communication

The TP53 Codon 72 Polymorphism May Affect the Function of TP53 Mutations in Breast Carcinomas but not in Colorectal Carcinomas[1]

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Abstract

An Arg/Pro polymorphism in codon 72 of the *TP53* gene was analyzed in blood samples from 390 breast and 162 colorectal cancer patients previously investigated for *TP53* mutations in their tumors. Among the breast cancer cases, 228 were homozygous for the Arg72 allele, of which, 65 (28.5%) also had a *TP53* mutation in their tumors. In contrast, of 26 cases that were homozygous for the Pro72 allele, only 1 case (3.8%) had a *TP53* mutation in the tumor ($P = 0.004$). Cloning the *TP53* gene from tumor DNA followed by sequencing was performed in 14 heterozygotes with tumor mutation, and 9 of the mutations resided on the Arg72 allele. Among the colorectal cancer cases, no difference in mutation frequency was seen between the two different homozygotes, 40 *TP53* mutations in 97 Arg72 homozygous cases (41.2%) *versus* 7 in 16 Pro72 homozygous cases (43.8%). These results suggest a selective growth advantage for cells carrying a type of *TP53* mutation seen in breast carcinomas when the mutation resides on an Arg72 allele.

Introduction

Mutations in the *TP53* gene are considered to represent the most common genetic alteration in human cancer. These mutations (mostly missense mutations) may damage the normal function of TP53 as a transcription factor, and the induction of repair or apoptosis may be abolished. Consequently, other genetic alterations may accumulate in the cell. In breast cancer, the *TP53* gene is mutated in ~20–30% of the tumors and in colorectal cancer in 50–60% [reviewed in Ref. 1].

In addition to gene mutations, several reports have focused on *TP53* polymorphisms as risk factors for malignant disease. Two of 14 known polymorphisms located in the *TP53* gene alter the amino acid (International Agency for Research on Cancer TP53 Mutation Database).[5] The alleles of the polymorphism in codon 72, exon 4, encode an arginine amino acid (CGC; Arg72) with a positive-charged basic side chain and a proline residue (CCC; Pro72) with a nonpolar-aliphatic side chain. Significant association between the codon 72 polymorphism and risk of cancer have been reported, although the results with regard to most cancer diseases, including breast (2–4) and colorectal carcinomas (5–7) remain inconclusive.

The Arg/Pro polymorphism is located in a proline-rich region (residues 64–92) of the TP53 protein, where the Pro72 amino acid constitutes one of five PXXP motifs resembling a SH3 binding domain. The region is required for the growth suppression and apoptosis mediated by TP53 but not for cell cycle arrest [reviewed in Ref. 1]. The two polymorphic variants of wild-type TP53 have been shown to have some different biochemical and biological properties (8) such as different binding to components of the transcriptional machinery and different activation of transcription, but they did not differ in their ability to bind DNA.

The TP73 protein, a homologue of the TP53 protein, is able to activate TP53-responsive promoters and induce apoptosis in TP53-deficient cells. Marin *et al.* (9) recently showed that some TP53 mutants can bind to and inactivate TP73 and that the binding of such mutants was enhanced when the mutation occurred on the Arg72 allele. They also reported a higher frequency of *TP53* mutations on the Arg72 compared with the Pro72 allele in different squamous cell cancers. These findings were supported by Tada *et al.* (10), which found an overrepresentation of mutations on the Arg72 allele in tumors from different tissues. Interestingly, they found a preferential selection of the Arg72 allele in cancers with recessive TP53 mutants (mutants that do not inactivate wild-type TP53 in a dominant negative manner). It was suggested that recessive TP53 mutants achieve a selective growth advantage by an Arg72-dependent inactivation of TP73, whereas the dominant negative TP53 mutants inactivate the remaining wild-type TP53 allele in an Arg72-independent manner.

We have investigated whether somatic *TP53* mutations exist in combination with a specific constitutional allele variant of the codon 72 polymorphism (Arg72 or Pro72) in a series of breast carcinomas and a series of colorectal carcinomas, which are known to have different *TP53* mutation spectrum.

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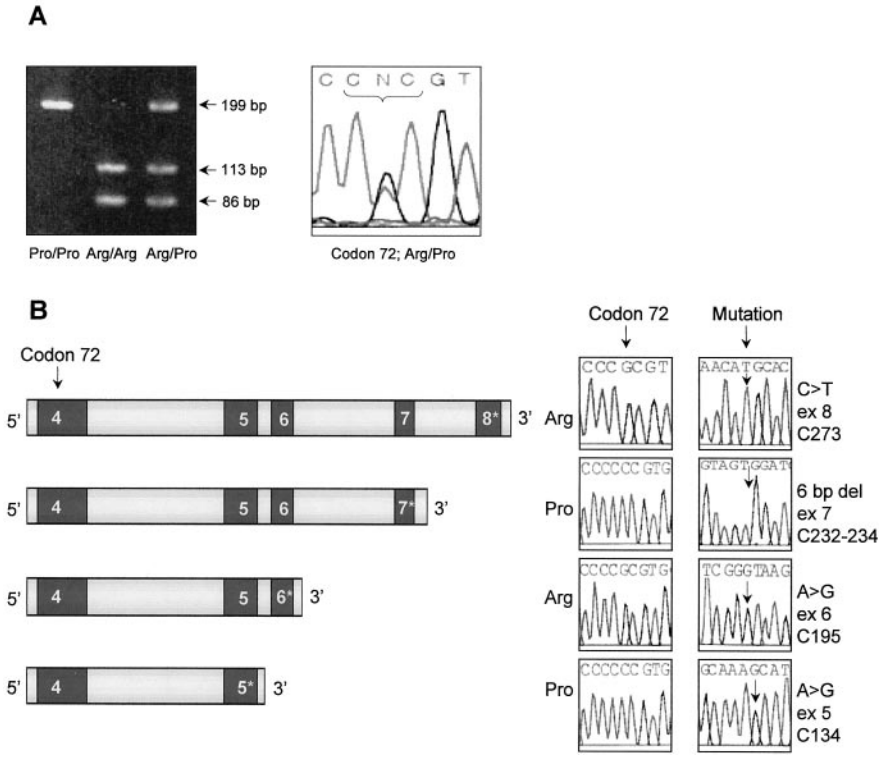

Fig. 1. *A*, the *TP53* codon 72 polymorphism was analyzed in DNA from blood using RFLP. Electrophoresis in 7.5% acrylamide gel gave an allele pattern of the two alleles of 113 bp +86 bp (Arg) and 199 bp (Pro). The gel shows three patients with different genotypes, along with the sequence of a heterozygous sample. *B*, from heterozygous samples (Arg72/Pro72) with a *TP53* mutation in their tumor, the *TP53* gene was cloned and sequenced to determine which allele the mutation resided on. Four different fragments were designed for cloning to include the polymorphism in exon 4 as well as the respective mutation (*, mutations located in exons 5–8). The part of the DNA sequence with the polymorphism and mutation are shown (mutations sequenced in reverse direction).

Materials and Methods

Materials. The study included 390 Norwegian breast cancer cases. These were from two different consecutive series (129 and 130 samples, respectively) previously described (11, 12) and from two series of advanced breast cancer cases (84 and 47, respectively), of which, one has been described previously (13). One hundred sixty-two Norwegian colorectal cancer cases previously analyzed for *TP53* mutations in their tumors were also included in this study (14). DNA had been isolated from both blood cells and tumor tissue using chloroform/phenol extraction followed by ethanol precipitation (Nucleic Acid Extractor 340A; Applied Biosystems) according to standard procedure.

Genotyping. DNA from blood samples was analyzed for the genetic variation in codon 72 in exon 4 of the *TP53* gene using Restriction Fragment Length Polymorphism analysis (15). Genomic DNA (50 ng) was amplified in 25 μl of PCR reactions (Eppendorf Mastercycler Gradient), containing 12.5 pmol of each primer (F: 5′-TTGCCGTCCCAAGCAATGGATGA-3′, R: 5′-TCTGGGAAGGGACAGAAGATGAC-3′), 2.5 μl of 10× buffer (Gene Amp from Applied Biosystems, containing 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% W/v gelatin), 10 mM deoxynucleotide triphosphate, and 0.75 units of AmpliTaq DNA Polymerase (Applied Biosystems). A 199-bp fragment was amplified using a PCR program starting with denaturation for 3 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 68°C, and 30 s at 72°C. Restriction analysis was performed mixing 8 μl of PCR product, 9 μl of H_2O, 2 μl of 1× NEBuffer 2, 1 μl of (10 units/μl) *Bst*UI (New England BioLabs), and incubated for 3 h at 60°C. Electrophoresis in 7.5% acryl amide gel gave an allele pattern of the two alleles of 113 bp +86 bp (Arg) and 199 bp (Pro), respectively (Fig. 1*A*).

Mutation Analysis. *TP53* mutation detection in tumor DNA was performed using Constant Denaturing Gradient Gel Electrophoresis (16) or Temporal Temperature Gradient Gel Electrophoresis (17). The samples from the two advanced breast cancer series (131 cases) have been screened for mutations in exons 2–11 of the *TP53* gene (13). One of the consecutive breast cancer series (130 cases) has been reanalyzed to include exons 2–11 (analysis of exons 5–8 reported in Ref. 12). The samples from the other consecutive breast cancer series (129 cases; Ref. 11), as well as the colon cancer cases (162 cases; Ref. 14) have been screened for *TP53* mutations in exons 5–8.

Table 1 Primers used for cloned inserts

Fragment	Primer
Exon 4–5 (1319 bp)	4F: 5′-gctgggggggctgaggacc-3′
	5R: 5′-gcaatcagtgaggaatcaga-3′
Exon 4–6 (1527 bp)	6R: 5′-ccactgacaaccaccctt-3′
Exon 4–7 (2217 bp)	7R: 5′-aggggtcagcggcaagcaga-3′
Exon 4–8 (2679 bp)	8R: 5′-aggcataactgcacccttgg-3′

Cloning and Sequencing. The cloning of the *TP53* gene from tumor DNA was performed using the TOPO TA Cloning kit (Invitrogen). Four different fragments were designed to comprise the polymorphism in exon 4, as well as the respective mutation (Fig. 1*B*). DNA (<50 ng) was amplified in 25 μl of PCR reactions (Eppendorf Mastercycler Gradient), containing 12.5 pmol of each primer (for primer sequences see Table 1), 2.5 μl of 10× buffer (Gene Amp; Applied Biosystems) giving a concentration of 1.5 mM Mg^{2+}, 10 mM deoxynucleotide triphosphate, and 0.75 units of AmpliTaq DNA Polymerase (Applied Biosystems). The PCR program started with denaturation for 2 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 63°C, 60 s at 72°C, and finally 10 min at 72°C. The PCR product was analyzed by gel electrophoresis (7.5% acrylamide)

Table 2 Overview of *TP53* mutations found

Characterization and distribution of *TP53* mutations in breast carcinomas with respect to genotype of the codon 72 polymorphism. The heterozygote samples that were cloned and sequenced to determine which allele of the codon 72 the mutation resided on are shown in bold and the respective allele underlined. (The sample IDs are designated MT and ULL for the two consecutive series, and LB and FU for the advanced breast cancer series.)

Sample ID	Genotype / Codon 72	Mutation / Exon	Codon	Codon change	Base change	Aminoacid	Type
ULL-T-271	Arg/Arg	3	29	1bp ins	ins A		Frameshift
ULL-T-177	Arg/Arg	4	110	CGT > CCT	G > C	Arg > Pro	Missense
LB 105 A	Arg/Arg	<5	1 bp upstream	Intronic	G > A		Splice
ULL-T-096	Arg/Arg	5		18 bp ins			In frame
ULL-T-038	Arg/Arg	5	138	GCC > GTC	C > T	Ala > Val	Missense
LB 101 A	Arg/Arg	5	151	CCC > TCC	C > T	Pro > Ser	Missense
MT 064	Arg/Arg	5	156	CGC > CCC	G > C	Arg > Pro	Missense
ULL-T-106	Arg/Arg	5	159	GCC > GAC	C > A	Ala > Asp	Missense
LB 703 B	Arg/Arg	5	168	CAC > CCC	A > C	His > Pro	Missense
ULL-T-099	Arg/Arg	5	173	GTG > CTG	G > C	Val > Leu	Missense
MT 193	Arg/Arg	5	174–180	17 bp del			Frameshift
FU M312	Arg/Arg	5	175	CGC > CAC	G > A	Arg > His	Missense
MT 083	Arg/Arg	5	175	CGC > CAC	G > A	Arg > His	Missense
ULL-T-171	Arg/Arg	5	175	CGC > CAC	G > A	Arg > His	Missense
LB 205 A	Arg/Arg	5	176	TGC > TTC	G > T	Cys > Phe	Missense
MT 101	Arg/Arg	5	181	CGC > CAC	G > A	Arg > His	Missense
FU 07	Arg/Arg	<6	3 bp upstream	Intronic	T > G		Splice
LB 307 B	Arg/Arg	6	190	CCT > CTT	C > T	Pro > Leu	Missense
MT 120	Arg/Arg	6	194	CTT > CGT	T > G	Leu > Arg	Missense
FU 23	Arg/Arg	6	195	ATC > ACC	T > C	Ile > Thr	Missense
ULL-T-179	Arg/Arg	6	196	22 bp del			Frameshift
FU M307	Arg/Arg	6	197–199	6 bp del			In frame
LB 111 A	Arg/Arg	6	204	GAG > TAG	G > T	Glu > Stop	Nonsense
MT 078	Arg/Arg	6	204	GAG > TAG	G > T	Glu > Stop	Nonsense
LB 805 A	Arg/Arg	6	213	CGA > TGA	C > T	Arg > Stop	Nonsense
ULL-T-188	Arg/Arg	6	216	GTG > ATG	G > A	Val > Met	Missense
FU M326	Arg/Arg	6	217	GTG > ATG	G > A	Val > Met	Missense
LB 123 A	Arg/Arg	6	217–221	14 bp del			Frameshift
MT 106	Arg/Arg	7	238	TGT > TTT	G > T	Cys > Phe	Missense
LB 305 A	Arg/Arg	7	239–242	11 bp del			Frameshift
MT 050	Arg/Arg	7	242	TGC > TTC	G > T	Cys > Phe	Missense
MT 359	Arg/Arg	7	242	TGC > TAC	G > A	Cys > Tyr	Missense
MT 135	Arg/Arg	7	244–247	8 bp del			Frameshift
MT 161	Arg/Arg	7	245	GGC > AGC	G > A	Gly > Ser	Missense
LB 309 A	Arg/Arg	7	248	CGG > CAG	G > A	Arg > Gln	Missense
MT 024	Arg/Arg	7	248	CGG > CAG	G > A	Arg > Gln	Missense
ULL-T-250	Arg/Arg	7	248	CGG > CAG	G > A	Arg > Gln	Missense
MT 160	Arg/Arg	7	248	CGG > TGG	C > T	Arg > Trp	Missense
LB 111 B	Arg/Arg	7	249	AGG > GGG	A > G	Arg > Gly	Missense
MT 071	Arg/Arg	7	251	ATC	del C		Frameshift
FU 27	Arg/Arg	7	256	12 bp ins			In frame
FU M327	Arg/Arg	8	261–269	24 bp del			In frame
LB 120 A	Arg/Arg	8	266	GGA > AGA	G > A	Gly > Arg	Missense
FU 26	Arg/Arg	8	273	CGT > CCT	G > T	Arg > Pro	Missense
LB 107 B	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
LB 208 A	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
LB 405 A	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
FU M310	Arg/Arg	8	273	CGT > CAT	G > LA	Arg > His	Missense
FU M321	Arg/Arg	8	273	CGT > TGT	C > T	Arg > Cys	Missense
MT 029	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
MT 240	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
ULL-T-164	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
ULL-T-071	Arg/Arg	8	273	CGT > GGT	C > G	Arg > Gly	Missense
LB 706 A	Arg/Arg	8	277	TGT > TAT	G > A	Cys > Tyr	Missense
ULL-T-215	Arg/Arg	8	278	CCT > CTT	C > T	Pro > Leu	Missense
MT 119	Arg/Arg	8	281	GAC > GGC	A > G	Asp > Gly	Missense
FU 12	Arg/Arg	8	282	CGG > GGG	C > G	Arg > Gly	Missense
MT 181	Arg/Arg	8	282	CGG > CAG	G > A	Arg > Gln	Missense
ULL-T-154	Arg/Arg	8	282	CGG > TGG	C > T	Arg > Trp	Missense
MT 003	Arg/Arg	8	285	GAG > AAG	G > A	Glu > Lys	Missense
LB 404 B	Arg/Arg	8	286	GAA > AAA	G > A	Glu > Cys	Missense
LB 406 A	Arg/Arg	<9	2 bp upstream	Intronic	A > G		Splice
FU M317	Arg/Arg	<9	2 bp upstream	Intronic	A > G		Splice
FU 04	Arg/Arg	>9	1 bp downstream	Intronic	G > C		Splice

Table 2 Continued

Sample ID	Genotype / Codon 72	Mutation / Exon	Codon	Codon change	Base change	Aminoacid	Type
FU M314	Arg/Arg	10	342	CGA > TGA	CST	Arg >Stop	Nonsense
ULL-T-263	Arg/Pro	4	89	11 bp ins			Frameshift
ULL-T-113	Arg/Pro	4	113	TTC > GTC	T > G	Phe > Val	Missense
MT 059	Arg/Pro	5	134	TTT > CTT	T > C	Phe > Leu	Missense
LB 709 B	Arg/Pro	5	136	CAA > TAA	C > T	Gln >Stop	Nonsense
FU 11	Arg/Pro	5	140–143	10 bp del		Stop169	Frameshift
FU M315	Arg/Pro	5	142	CCT	del C	Stop169	Frameshift
MT 052	Arg/Pro	5	156	CGC > CCC	G > C	Arg > Pro	Missense
LB 115 B	Arg/Pro	5	163	TAC > TGC	A > G	Tyr > Cys	Missense
LB 708 B	Arg/Pro	5	165	CAG > TAG	C > T	Gln >Stop	Nonsense
FU M304	Arg/Pro	5	167	CAG > TAG	C > T	Gln >Stop	Nonsense
MT 022	Arg/Pro	5	172	GTT	del T		Frameshift
MT 020	Arg/Pro	5	174–180	17 bp del			Frameshift
ULL-T-155	Arg/Pro	5	175	CGC > CAC	G > A	Arg > His	Missense
MT 208	Arg/Pro	5	179	CAT > TAT	C > T	His > Tyr	Missense
FU 05	Arg/Pro	6	195	ATC > ACC	T > C	Ile > Thr	Missense
MT 111	Arg/Pro	6	195	ATC > ACC	T > C	Ile > Thr	Missense
ULL-T-007	Arg/Pro	6	195	ATC > ACC	T > C	Ile > Thr	Missense
FU M316	Arg/Pro	6	220	TAT > TGT	A > G	Tyr > Cys	Missense
ULL-T-226	Arg/Pro	6	220	TAT > TGT	A > G	Tyr > Cys	Missense
LB 303 B	Arg/Pro	7	232–234	6 bp del			In frame
LB 206 A	Arg/Pro	7	237	ATG > ATT	G > T	Met > Ile	Missense
MT 104	Arg/Pro	7	239	AAC > ACC	A > C	Asn > Thr	Missense
FU M303	Arg/Pro	7	248	CGG > TGG	C > T	Arg > Trp	Missense
MT 112	Arg/Pro	7	248	CGG > TGG	C > T	Arg > Trp	Missense
MT 132	Arg/Pro	7	248	CGG > CAG	G > A	Arg > Gln	Missense
MT 016	Arg/Pro	8	273	CGT > CAT	G > A	Arg > His	Missense
MT 065	Arg/Pro	8	273	CGT > CAT	G > A	Arg > His	Missense
FU M301	Arg/Pro	8	282	CGG > GGG	C > G	Arg > Gly	Missense
MT 318	Arg/Pro	8	282	CGG > TGG	C > T	Arg > Trp	Missense
FU 06	Arg/Pro	8	298	GAG > TAG	G > T	Glu >Stop	Nonsense
LB 122 A	Pro/Pro	<5	1 bp upstream	Intronic	G > A		Splice

for quality check, then cloned into the pCR 2.1-TOPO vector and transformed into *Escherichia coli* according to standard protocols. The plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen), and the complete insert was sequenced (ABI 3100; Applied Biosystems) in overlapping fragments.

Statistics. Deviations from Hardy-Weinberg equilibrium of the codon 72 polymorphism were determined using χ^2 test. Cross-tabulation and χ^2 test were performed when studying the polymorphism's association with *TP53* mutations. Pearson χ^2 test or Fisher's exact test (when appropriate) was used, and statistical significance level was set to $P \leq 0.05$. Computations were performed using Excel (Microsoft Excel 97) and SPSS (version 8.0).

Results and Discussion

Among the 390 breast cancer cases genotyped, the allele frequencies were 0.76 and 0.24 for the Arg72 and Pro72 allele, respectively, and the polymorphism was shown to be in Hardy-Weinberg equilibrium. The *TP53* mutation frequencies in the different series, where screening of exons 2–11 were performed, were 14.6% (19 of 130) in the consecutive series and 28.6% (24 of 84) and 46.8% (22 of 47) in the two series of advanced breast cancer cases, reflecting the different distribution of tumor size and stage of disease between these series. Of the 228 cases that were homozygous for the Arg72, 65 (28.5%) also carried a *TP53* mutation in their tumors. In contrast, of the 26 cases that were homozygous for the Pro72 allele, only 1 case (3.8%) had a *TP53* mutation (Table 2). Thus, the occurrence of a *TP53* mutation was significantly more often found on the Arg72 allele than the Pro72 allele ($P = 0.004$). This skewed distribution was seen in all series, although each of them was too small to give significant results by their own. When limited to *TP53* mutations residing in exons 5–8, the same significant biased distribution was seen ($P = 0.007$). Only 8 of 65 mutations (12.3%) were located outside exons 5–8, of which, 6 were found in Arg72 homozygous and 2 in heterozygous (Table 2). The same skewed distribution with respect to genotype was also seen when considering only missense mutations. Of the 228 homozygotes for the Arg72 allele, 45 (19.7%) missense mutations were found, whereas none of the 26 homozygotes for the Pro72 allele carried a missense mutation ($P = 0.006$).

From 14 heterozygotes (Arg72/Pro72) with a *TP53* mutation in their tumor and where enough tumor DNA still was available, the *TP53* gene was cloned and sequenced to determine which allele the mutation resided on (Fig. 1*B*). In 9 cases, the mutations (5 missense, 2 nonsense, and 2 frameshifts) were located on the arginine allele, and in the 5 remaining samples, the mutations (2 missense, 1 frameshift, and 1 deletion) resided on the Pro allele supporting the findings in the homozygous samples (Table 2).

The observed skewed occurrence of somatic *TP53* mutations on the Arg72 allele in breast carcinomas suggests that this combination gives breast epithelial cells a growth advantage, which may increase the risk of malignant transformation and development of cancer. The coexistence of the Arg72 with a mutation may modify the TP53 protein structure in a way that

interferes either with the protein's ability to achieve sequence-specific binding to DNA or with the interaction and recruitment of the transcription machinery, causing an altered transcription pattern (18). Another possibility is that the Arg72 may modify the mutant TP53 protein's ability to bind to and interact with other proteins such as, for example, TP73. Interaction between tumor-derived TP53 mutants and TP73 has been observed (19), and the codon 72 polymorphism has been reported to be a modifier of such an interaction (9), which may interfere with TP73-induced apoptosis.

The same level of skewed distribution of mutations residing on the Arg72 as seen for all type of mutations was also seen for missense mutations, giving no evidence for a stronger effect of such mutations. However, missense mutations are of many different types, and classifications according to structure or function in different cell types in larger series may give other results. The more severe changes like deletion, insertion, nonsense, and splice mutations may lead to a truncated protein or lack of protein where a codon 72 polymorphism has no modifying impact. Analyzing nonmissense mutations as one group with respect to the codon 72 homozygotes gave no skewed distribution ($P = 0.710$). The number is, however, small, and even truncated TP53 proteins may have an impact through mechanisms like inactivating other proteins (*e.g.*, TP73) if their interacting domain is intact and the protein is stable. It cannot be excluded that the two polymorphic variants may have different effects also on such mutants.

Genotyping of the 162 colorectal cancer cases revealed allele frequencies of the Arg72 and Pro72 alleles of 0.75 and 0.25, respectively. The *TP53* mutation frequency in this cohort was 48.1%. In contrast to the breast cancer cases, no difference in the frequency of mutations between the two different homozygotes was found in the colorectal cancer cases, with 40 *TP53* mutations in 97 Arg72 homozygous cases (41.2%) *versus* 7 *TP53* mutations in 16 Pro72 homozygous cases (43.8%). The spectrum of mutations is different between these two tumor types,[6] partly attributable to tissue-specific differences in carcinogen exposure and in metabolism (reviewed in Ref. 1). Breast cancer is reported to have a high level of insertions, deletions, and nonsense mutations, and GC:AT transitions are the most frequent change, equally distributed between CpG and non-CpG areas, whereas colorectal cancer has a high frequency of CpG transitions leading to mutants with a presumable dominant negative effect (1). A recent report divided the mutations into two groups according to their predicted dominant negative or recessive characteristics based on the results of a transactivation assay (9), and the authors proposed that it was only the recessive mutations that preferentially was located on the Arg72 allele (10). The dominant negative mutants were suggested to be independent of the codon 72 polymorphism. Using the same criteria for classifying the mutations as proposed by Tada *et al.* (10) on our series, the frequency of dominant negative mutants were higher in the colorectal cancer cases (83.3%, 30 of 36) than in the breast cancer cases (61.1%, 22 of 36; $P = 0.064$). Although only a minor fraction of our mutants (72 of 175) could be classified according to these categories, these results nevertheless support the hypothesis that a tumorigenic effect of the Arg72 allele only occurs when combined with a somatic mutation of the type seen in breast carcinomas. Additional studies, including functional assays, are warranted to explore the effects of the different combined variants and their role in tumorigenesis in different tissues.

[6] Internet address: www.iarc.fr/P53/index.html.

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