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THE ROLE OF *ATM* IN BREAST CANCER

Ph.D. Basic Theses

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1. INTRODUCTION

1.1 The *ATM* Gene

DNA repair is a crucial physiological mechanism maintaining genomic DNA integrity in each nuclear cell. The ataxia telangiectasia mutated (ATM) protein belongs to the core components of DNA damage response machinery and acts as an intracellular sensor by recognizing DNA double strand breaks (DSB). Numerous substrates involved in DNA repair are regulated by ATM protein kinase activity, thereby enabling DNA breaks to be linked with the processes of cell cycle and/or apoptosis.

When mutated, the *ATM* gene is the underlying cause of the rare autosomal recessive disorder ataxia telangiectasia (AT) characterized by cerebellar ataxia, oculocutaneous telangiectasia, immunological deficiency, hypersensitivity to ionizing radiation and predisposition to cancer [1]. More than 400 disease-causing mutations have been identified in *ATM* thus far and more than 70% of them result in premature termination of translation and truncation of the protein [2,3]. The typical AT phenotype is caused by *ATM* null alleles that either truncate (frameshift or nonsense mutations) or severely destabilize (mutations affecting mRNA splicing) the ATM protein [4,5]. Most mutations are unique and uniformly distributed throughout the gene´s open reading frame with no apparent hotspots. While the penetrance of truncating mutations may approach 100%, penetrance of missense mutations is lower and often results in a milder phenotype [6]. Usually, AT patients are compound heterozygotes; patients homozygous for the same mutation are rare.

The *ATM* gene was mapped to chromosome 11q22-23 in 1988 [7] and cloned in 1995 [8]. The entire gene spans almost 150 kb of the genomic DNA, consists of 66 exons and is transcribed in a wide range of tissues to an mRNA of approximately 13 kb with a coding sequence of 9168 bp. The gene encodes the 350 kDa protein containing 3056 amino acid residues with serine-threonine protein kinase activity [9].

ATM and ATM and Rad3 related kinase (ATR) are thought to be master controllers of cell cycle checkpoint signaling pathways required for cell response to DNA damage and for genome stability. Besides being present in the nucleus, the ATM protein is located in the cytoplasm. Intracellular ATM concentration is not altered by radiation exposure but its innate kinase activity increases severalfold after exposure to IR [10].

1.2 ATM Function

ATM is the initiator of a signaling cascade that responds to DSB. Normally, ATM is present in cells in the form of an inactive complex, either a dimer or a higher multimer. Following DNA damage, ATM undergoes autophosphorylation at S1981 leading to the dissociation of inactive complex and subsequent triggering of a signaling cascade involving phosphorylation of several substrates [11], which, in turn, results in two crucial responses to DNA damage: the activation of cell cycle checkpoints and the initiation of DNA repair. However, DNA repair failure may result in the activation of apoptosis. ATM substrates include p53, Mdm2 and c-Abl influencing the G1 checkpoint; NbsS1, Rad51, BRCA1 and Fanconi anemia complementation group D2 protein (FANCD2) involved in the transient IR-induced S-phase arrest, and BRCA1 and checkpoint kinases 1 and 2 (Chk1, Chk2) regulating the G2 checkpoint [11] (see figure 1). Simultaneously, the ATM activity can be modulated by some of its substrates. Physiological DSB occurring during meiosis and immune system maturation also require ATM to initiate recombinational DNA repair.

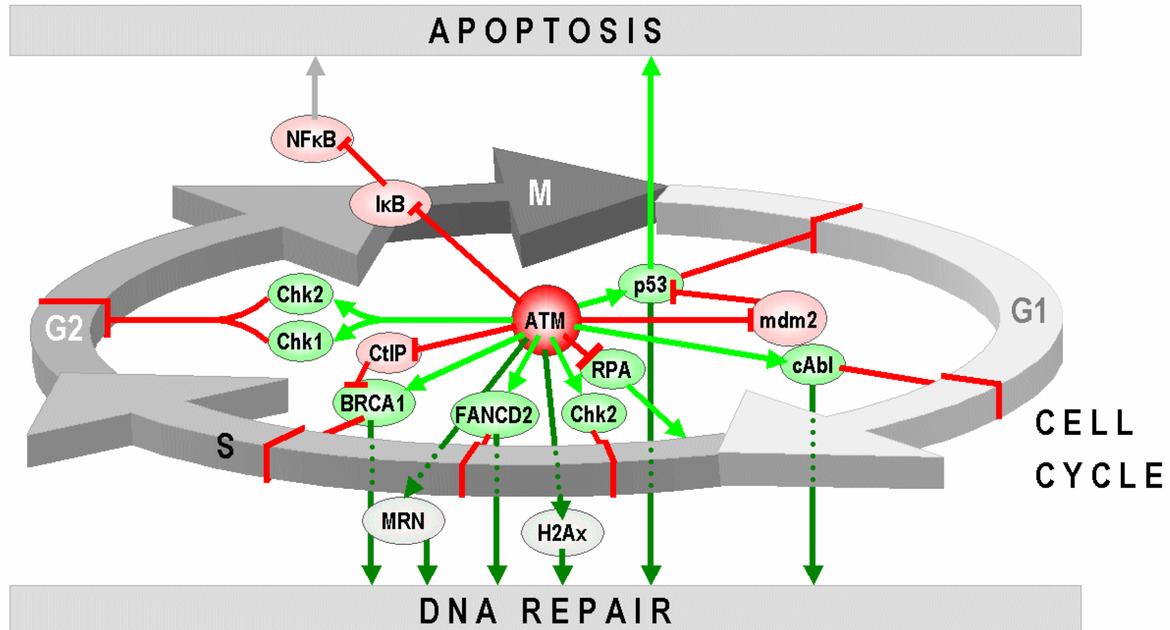

Figure 1: *ATM is the key player in response to DNA double strand breaks. ATM can control cell cycle through phosphorylation of several substrates such as p53, cAbl, BRCA1 and Chk1/2. ATM induces DNA repair via activation of several proteins, e.g. H2AX, MRN complex (Mre11,Rad50, Nbs1) or BRCA1. ATM can regulate apoptosis for example through IκB phosphorylation. However, its role in apoptosis is not well understood. For details, see [12]*

1.3 *ATM* and Cancer

During their shortened lifetime, nearly 40% of AT homozygotes develop a malignancy [13]. The tumor types in AT are primarily lymphomas or leukemias (approximately 85%) as the ATM protein in the AT individuals is unable to repair the normal DNA breakage generated during the immune system maturation [14].

The heterozygous carriers of *ATM* mutations account for 0.35–1% of the general population and their life expectancy is reduced because of an increased appearance of age-related disorders such as ischemic heart disease [15]. With respect to the central role of ATM in the DNA damage response, it was expected that carriers would have an increased risk of various cancers. However, the elevated incidence of ovarian, lung, gastric, pancreatic, colon or biliary system cancers was not detected in AT families [16]. Interestingly, there is a correlation with *ATM* heterozygotes and an increase in breast cancer risk [17].

1.4 *ATM* and Familial Breast Cancer

Breast cancer is the most common malignancy affecting western women with the lifetime risk of approximately 10-12%. The most significant risk factor is a family history of breast cancer. About 5-10% of all breast cancer cases are due to germline mutations in high penetrance genes. In the majority of affected families (60-80%), the disease is linked to the two major susceptibility genes, *BRCA1* and *BRCA2* [18]. Mutations in other genes such as *Chk2* and *ATM* are good candidates for risk factors as well.

The initial interest in studying *ATM* heterozygosity arose from epidemiological studies in 1991 indicating an increased risk of breast cancer in obligate AT heterozygotes from American families. In this study the relative risk of breast cancer in women was estimated to be approximately 5 times as high as in control subjects [19]. However, *ATM* mutation screening studies in cancer patients brought controversial results about the degree of the risk for breast cancer in heterozygous carriers of *ATM* mutations. In the study of Broeks *et al.*, truncating germline mutations in *ATM* were identified in 7/82 (8.5%) Dutch patients with early onset breast cancer, and *ATM* heterozygotes had an approximately ninefold increased risk of breast carcinoma development [20]. Approximately twofold increase in risk of breast cancer was associated with *ATM* mutations in Britain [16,21]. Thorstenson *et al.* found 7 AT-causing mutations in 230 (2.6%) high-risk breast and ovarian cancer families in Austria [22]. Recently, Renwick *et al.* concluded that women heterozygous for mutations causing AT in homozygotes have approximately twofold increase in risk of breast cancer development [23].

Nevertheless, many studies have failed to confirm the role of AT-causing mutations in breast cancer development and concluded that their contribution to familial breast cancer is minimal [24,25]. The studies that were focused on early onset or increased radiosensitivity breast cancer failed to provide evidence of increased frequency of *ATM* mutations compared to control subjects [26,27]. Other studies found a higher rate of amino acid substitutions in ATM in patients with breast cancer [25,28]. However, the significance of most missense variants is unknown and only a few of them have been considered to be pathogenic. The c.7271T>G transversion at the codon 2424, leading to the replacement of valine with glycine and consequently to the ATM protein with residual function, was associated with a mild clinical phenotype and lower radiosensitivity in homozygotes and increased risk of breast cancer in both the homozygotes and the heterozygotes [2]. The IVS10-6T>G splice site mutation causing the loss of exon 11 in the *ATM* mRNA and reduction of the full-length mRNA and ATM protein, is a controversial missense mutation. Broeks *et al.* concluded that heterozygous carriers of this mutation have an increased risk of breast cancer development characterized by increased occurrence of bilateral cancer and early age at onset [29]. However, Szabo *et al.* reported that IVS10-6T>G did not confer an elevated risk for breast cancer [30].

It has been proposed that heterozygotes with the truncating type of mutations and those with missense mutations can exhibit distinct cancer risk as both types of mutations can exert variant effects on ATM function. Little or no protein, even a shortened form, is produced from the allele with the truncating type of mutation. On the other hand, alleles with the missense type of mutation express an ATM protein, albeit of a mutant form. According to this hypothesis, the truncating mutations act in recessive fashion whereas most missense mutations may act in a dominant fashion [6]. Funtional assays are necessary to evaluate the true significance of particular mutations in breast cancer development.

It has been shown *in vitro* that cells heterozygous for AT-causing mutations are more sensitive to radiation [31]. Additionally, irradiated mice heterozygous for *ATM* mutations had an increased frequency of dysplastic breast cells, supporting an increased cancer risk for heterozygotes that is related to their mutagene exposure [32]. The consequences of these studies are not merely academic as a common treatment regime for breast cancer involves radiotherapy. Paradoxically, could part of the cure exacerbate the disease in breast cancer patients who are *ATM* heterozygous? Also mammography involves irradiation, so could breast screening provoke breast cancer development in *ATM* heterozygous women? So far, human studies with small numbers of patients have not found a correlation between hyperradiosensitivity in breast cancer patients treated by radiotherapy and mutations in the

ATM gene [20], but much more research is needed. Until it has been proven safe, perhaps non-radiation techniques for the early detection of breast cancer, such as sonography or magnetic resonance imaging, should be used and radiotherapy given as a last resort in *ATM* heterozygotes.

1.5 *ATM* and Sporadic Breast Cancer

Breast cancer is a malignancy with numerous genetic alterations. The classical Knudson´s two hit hypothesis predicted that a phenotypic manifestation of tumor-suppressor gene inactivation is undetectable unless the two alleles of a single gene are inactivated. Alleles of tumor-suppressor genes can be lost or inactivated in a variety of ways during tumour development, ranging from chromosomal deletions, insertions or rearrangements to single base mutations or epigenetic promoter alterations such as DNA hypermethylation.

Tumor-suppressors often reside in genomic regions characterized by frequent chromosomal deletions, which cause loss of heterozygosity (LOH). Generally, the background rate of allelic deletions reaches up to 5% [33]. However, deletions of *ATM* alleles are much more common in breast tumors indicating the important role of the gene in breast cancer development. Numerous studies identified allelic losses in nearly 40% of sporadic breast cancer cases and suggested that LOH in the *ATM* locus may occur early in breast tumorigenesis [33,34]. Nevertheless, the process of the second allele inactivation often remains unidentified. Some findings indicated that haploinsufficiency at ATM may promote tumorigenesis due to the increased chromosomal instability, even though LOH identification at the *ATM* loci supported the more classic two-hit tumor-suppressor gene model [35]. In recent epidemiologic studies, no somatic mutations were found in mammary carcinomas from midwestern United States and Japan [36]. Thus, it seems that inactivation of the *ATM* gene by somatic mutations is not a common hallmark of breast cancer. Similar results were obtained from BRCA1/2 studies, where acquired somatic mutations rarely occur in these major breast cancer susceptibility genes [37].

Another mechanism contributing to the loss of a tumor-suppressor function is the methylation of cytosine residues located within the CpG islands. These commonly unmethylated dinucleotide regions are clustered in the proximal promoters of almost half of the genes in the human genome [38]. Promoter hypermethylation often results in an inappropriate transcriptional silencing of nearby genes and is at least as common as the disruption of classic tumor-suppressor genes in human cancer by mutations [38]. In cancer,

many key tumor-suppressor genes, including *BRCA1* and *E-cadherin*, are subjected to this epigenetic silencing, which is now widely recognized as a causative or correlative event in tumor development. Nearly 50% of the tumor-suppressor genes associated with rare inherited cancer syndromes are known to undergo methylation of their promoters in sporadic cancer [38]. Numerous studies demonstrated the hypermethylation as a second inactivating event in tumors in patients carrying germline mutations in tumor-suppressor genes. Interestingly, methylation seems to be always associated with the wild-type allele [38].

ATM is transcribed from a bidirectional promoter. The 520 bp intergenic region is shared with the *NPAT* gene. NPAT protein is a substrate of cyclin E-Cdk2 kinase and is thought to play an important role in a coordinated transcriptional activation of histone genes during the G1/S phase transition and in S phase entry in mammalian cells [39]. Nevertheless, the *ATM* promoter methylation was found not to influence the *NPAT* gene expression [40]. The epigenetic silencing of *ATM* expression was described once in locally advanced breast tumors suggesting a link between reduced ATM function and sporadic breast cancer [41]. However, other studies did not reveal hypermethylation of *ATM* promoter in any of tested breast tumor samples [42,43]

2. AIMS OF THE STUDY

The aim of this study was to analyze the *ATM* gene in breast cancer including the design and optimalization of particular methods. The study was focused on the following parts:

1. The role of the *ATM* gene in hereditary breast cancer

- Mutation analysis of the *ATM* gene in high-risk breast cancer patients
- Analysis of breast tumor tissue gained from patients carrying mutation in the *ATM* gene

2. The role of the *ATM* gene in sporadic breast cancer

- Loss of heterozygosity analysis in sporadic breast cancer
- Mutation analysis of the *ATM* gene in sporadic breast cancer
- Detection of *ATM* promoter hypermethylation in sporadic breast cancer

3. MATERIALS AND METHODS

3.1 Material

3.1.1 Hereditary breast cancer

EDTA blood samples were collected from high-risk breast cancer patients at the Department of Oncology and at the Department of Gynecology and Obstetrics of the First Faculty of Medicine of Charles University in Prague. A total of 114 patients were selected from families with two cases of breast cancer diagnosed before the age of 50 or three or more cases of breast (or ovarian) cancer diagnosed at any age. Genetic testing was further offered to patients diagnosed with breast cancer before the age of 40 (42 women) or with bilateral breast cancer before the age of 50 (5 women), regardless of family history of cancer. The paraffin-embedded tumor samples from ATM mutation carriers were obtained from the Institute of Pathology of the First Faculty of Medicine. Control blood samples were obtained from random blood donors of mixed gender. All women in the study gave signed informed consent prior to genetic testing. The protocol of investigation was approved by the Ethical Committee at the First Faculty of Medicine.

3.1.2 Sporadic breast cancer

73 nonselected sporadic tumors paired with EDTA blood samples were collected at the Department of Oncology and at the Department of Gynecology and Obstetrics of the First Faculty of Medicine of Charles University and General Teaching Hospital in Prague . All tumors were histologically confirmed and analysed for grading, estrogen (ER) and progesteron (PR) receptors, c-ErbB-2, Ki-67 and proportion of cancer cells.

3.1.3 DNA and RNA isolation

Genomic DNA was isolated from blood samples using the Wizard Genomic DNA Purification Kit (Promega) and from tumor samples using the QIAamp DNA Mini Kit (Qiagen). Total RNA from frozen tissue was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer´s instructions. Total RNA from peripheral blood lymphocytes was purified by acid guanidinium thiocyanate- phenol-chlorophorm extraction [44] and reverse-transcribed into cDNA using random hexamers according to the manufacturer’s protocol (Roche).

3.2 Mutation analysis

Mutational screening, based on RNA-analysis, included amplification of the entire coding sequence of the *ATM* gene, pre-screening of mutations by the protein truncation test (PTT) and their characterization by direct DNA sequencing. The coding region of *ATM* was divided into 7 overlapping fragments and amplified from cDNA by the polymerase chain reaction (PCR). Amplifications were performed in 10 µl reaction mixtures containing 1 µl of PCR buffer (Roche Molecular Biochemicals), 0.2 mM of each dNTP, 0.4 µM of each primer, 1 µl of cDNA template and 0.5 U of Fast Start Taq DNA polymerase (Roche). Following initial denaturation (at 95º C for 6 min), 35 cycles (at 95ºC for 30 sec, 58ºC or 62ºC for 30 sec for individual fragments, and 72ºC for 2 min) and final extension (at 72ºC for 7 min) were performed. PCR products were analyzed on 1.5% ethidium bromide-stained gels. Primer sequences were taken from Telatar *et al.* [45]. From each sample, 1 µl of the PCR product was incubated in the TnT/T7 coupled transcription/translation system (Promega) supplemented with [^{35}S]methionine (Amersham Biosciences) following the manufacturer's protocol and translated proteins were then separated on 12% SDS-polyacrylamide gels. After electrophoresis, gels were fixed, washed in fluorographic solution (Amplify, Amersham Biosciences), dried and exposed to X-ray film at -80°C. PCR products that gave rise to truncated proteins by PTT-analysis were purified and directly sequenced using the BigDye terminator cycle sequencing kit version 3.1 in a model AbiPrism 310 or 3130 automated DNA sequencer (Applied Biosystems). Mutations were confirmed by nucleotide sequence analysis of DNA samples.

The nomenclature of mutations used is according to den Dunnen and Paalman [46], with nucleotides numbered from the A of the ATG initiation codon of GenBank reference sequence U33841 for the complete *ATM* cDNA. Original designations for *ATM* mutations commonly referred to in the literature are included for ease of cross-referencing.

3.3 Analysis of splicing products in tumors with *ATM* germline mutation

To specify precisely the effect of IVS10-6T>G, IVS36+1A>G and c.6096-9delTTCTT mutations detected in high-risk breast cancer patients on splicing, we analyzed RNA samples extracted from peripheral blood of heterozygous mutation carriers and control healthy individuals. Individual cDNA samples obtained by reverse transcription served as templates for PCR-amplification using primers flanking either exon 11 (5′-GATCTGCTAGTGAATGAGATAAGTC-3′ - forward and 5′-AATGCAACTTCCGTAAGGC-3′ -

reverse) or exon 36 (5′-CCATTGACAAGACTTGAAGGACT-3′ - forward and 5′-GCTGTAGATAGGCCAGCATTG-3′ - reverse) or exon 44 (5′-CAGAAGTATAGGGGAGCCAGATAG-3′ - forward and 5′-CTGACGGAAGTGCAATGGT-3′ - reverse). PCR reactions were performed in 27 cycles (annealing at 60ºC, 58ºC and 64ºC, respectively); other reaction conditions and cycling parameters were as described above. The forward primers were fluorescently labeled with 6-FAM. The PCR products were separated in an ABI 3130 automated DNA sequencer and relative quantities of the full-length products and the products lacking the respective exon were determined by GeneMapper Software Version 4.0 (Applied Biosystems).

3.4 Immunohistochemistry

Paraffin sections obtained from tumor-tissue or metastasis of heterozygous mutations carriers as well as from sporadic tumors were used for histological and immunohistochemical examination. The mouse monoclonal antibody ATM Ab-8 (clone ATX08) (Neomarkers – Lab Vision Corporation) specific to the C-terminus of ATM was used for the detection of the protein according to the manufacturer's instructions.

3.5 LOH analysis

Paired samples of lymphocyte and tumor DNA served for LOH analysis carried out for three microsatellite markers; one (D11S2179) intragenic to *ATM* and two (D11S1819, D11S1294) that flank this gene on the centromeric and telomeric side, respectively. Primer sequences for microsatellite marker loci were retrieved from the Genome Database (http://www.gdb.org). PCR reactions of 10 µl volume contained 0.5 U of TaqDNA polymerase (Finnzymes) and 20-50 ng of DNA. Amplification was carried out under the following conditions: denaturation at 93°C for 3 min; 35 cycles of 93°C for 30 sec, 55°C, 60°C or 65°C (for D11S1294, D11S1819 and D11S2179, respectively) for 30 sec and 72°C for 30 sec; final extension at 72ºC for 5 min. PCR products were analyzed either on precast Spreadex gels or on automatic sequencer AbiPrism3130.

3.5.1 Electrophoresis on precast Spreadex gels

PCR products were run on precast Spreadex gels using SEA 2000 electrophoretic apparatus (Elchrom Scientific) and visualized by SYBR Gold Nucleic Acid Gel Staining (Invitrogen) according to manufacturer's instructions. The ElQuant software (Elchrom Scientific) was used for choosing an appropriate gel and electrophoretic conditions. Two-fold

or greater reduction in the relative allele intensity ratio measured between tumor and normal DNA was indicative of LOH for a given marker. Assessment of allele loss was accomplished by two independent observers.

3.5.2 Electrophoresis on AbiPrism 3130

Prior this analysis, the PCR products were amplified using forward primers labelled on 5´end by 6-FAM. 0.5 µl PCR product was mixed with 9.3 µl HiDi formamide and 0.2 µl size standard LIZ-600 (Applied Biosystems) and denaturated. Elektrophoresis was performed under denaturing conditions in the 36 cm capillary filled with POP-7 polymere on Abi Prism 3130 (Applied Biosystems). GeneMapper, v4.0 (Applied Biosystems) was used for the LOH assessment.

3.6 Promoter methylation analysis

Hypermethylation of *ATM* promoter was analysed by bisulfite sequencing and methylation specific multiplex ligation-dependent probe amplification (MS-MLPA).

3.6.1 Bisulfite sequencing

The DNA isolated from tumor tissue was analyzed for the cytosin methylation status of the *ATM* promoter by bisulfite sequencing. DNA samples (0.5 µg of each) were modified by bisulfite using EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's protocol. CpGenome Universal Methylated DNA (Intergen) was used as a positive control. The PCR mixture contained 1x PCR Gold Buffer, 2 mM MgCl2, 0.4 mM of each dNTP, 0.4 µM of each primers (5´-TTTTTAGATTTGGAGGGG-3´ - forward and 5´-CCCACTTCTAAAAAAAAAAA-3´ - reverse), 2 µl of bisulfite-modified DNA and 0.625 U of hot-start AmpliTaq Gold polymerase (Applied Biosystems) in a total volume of 25 µl. Amplification was performed under the following conditions: 95°C for 10 min and 40 cycles of 95°C for 30 sec, 52°C for 30 sec and 72°C for 40 sec. The PCR products were directly sequenced using the forward primer and the BigDye terminator cycle sequencing kit version 3.1 in a model AbiPrism 3130 automated DNA sequencer (Applied Biosystems).

3.6.2 MS-MLPA

Two kits - MS-MLPA ME001 and ME002 (MRC Holland) together with methylation sensitive endonuclease HhaI (Promega) were used for detection of the *ATM* promoter methylation. Amount of 90 ng of tumor DNA was used as a template. CpGenome Universal Methylated DNA served as a control (Intergen). Reactions were performed in half volume according to the manufacturer´s instructions (for further information see www.mlpa.com).

Electrophoresis was performed under denaturing conditions in the 36 cm capillary filled with POP-7 polymere on Abi Prism 3130 (Applied Biosystems). Promoter methylation was connected with presence of corresponding peak and was quantified through comparison of ratio between tested peak and sum of control peaks in reaction with and without added HhaI nuclease. Methylation higher than 10% was assessed as abberant [47].

4. RESULTS AND DISCUSSION

4.1 ATM and hereditary breast cancer

In our study, pathogenic mutations in *ATM* were detected in 3 (1.9%) out of 161 high-risk breast cancer patients not related to *BRCA1/2* whereas none mutation was identified in 183 controls. Two detected pathogenic mutations were splicing variants resulting in a shift of the *ATM* reading frame and premature termination of translation. The incidence of truncating mutations predominating in AT was comparable to that ascertained in UK (9/443, 2.0%) [23] and Austria (4/270, 1.5%) [22]. In addition, we detected the splicing variant c.1066-6T>G once among affected individuals (1/161; 0.6%) and twice among controls (2/183; 1.1%) (Tables 1 and 2). To investigate the effect of mutations intervening in splicing, we performed quantitative analyses of RNA samples obtained from breast cancer patients carrying the respective mutations in *ATM* and from unaffected healthy individuals.

Table 1. Frequencies of ATM mutations in relation to the classification of patients

Classification	No of patients/controls	No. of mutations exluding c.1066-6T>G (%)	c.1066-6T>G (%)
Breast cancer patients			
from HBC/HBOC families	114	2 (1.8)	1(0.9)
without family history of breast cancer			
bilateral breast cancer before 50	5	0 (0)	0
breast cancer before 40	42	1 (2.4)	0
Total	**161**	**3 (1.9)**	**1 (0.6)**
Controls	183	0	2 (1.1)

HBC, hereditary breast cancer; HBOC, hereditary breast and ovarian cancer

The c.5932G>T nonsense mutation that leads to the termination of translation at codon 1978 had previously been shown as causative in AT [5]. The mutation c.6096-9delTTCTT (IVS43-9del5), described in AT patients, causes skipping of the adjacent exon 44 as a result of the disturbance of the polypyrimidine tract close to the 3´splice site [5]. The c.5177+1G>A (IVS36+1G>A) mutation affects the 5´splice site, which results in the aberrant splicing and loss of exon 36. Quantitative analysis showed that the shortened transcript with the loss of exon 36 represented 53.6% of the *ATM* transcripts on average in a heterozygous carrier of this mutation. Similar results of RNA analysis (47.2% of the spliced transcript lacking exon 44 on average) were obtained in a carrier of the splicing mutation c.6096-9delTTCTT that was confirmed to cause AT. No transcripts lacking exon 36 or 44, respectively, were observed in

controls. Presented data indicate that the c.5177+1G>A mutation can be predicted to cause AT.

The c.1066-6T>G (IVS10-6T>G) splicing variant also affects the highly conserved polypyrimidine region and leads to incorrect splicing and loss of exon 11. The substitution has previously been identified in a number of breast cancer patients, however only once in an AT patient in homozygous state [29]. In the study of Szabo *et al.*[30], similar frequencies of the c.1066-6T>G mutation were found in a group of breast cancer families from diverse geographical regions obtained from five centers (8/961; 0.8%) and in a group of control individuals (4/543; 0.7%). Neither Bernstein *et al.* found an association between c.1066-6T>G and breast cancer [48]. In our study, incorrect splicing and loss of a small proportion of exon 11 (6%-15%) was also detected in control individuals without the c.1066-6T>G mutation in both lymphocytes and breast tissue. Presented results suggest that the variant c.1066-6T>G does not confer an elevated breast cancer risk. On the other hand, individuals heterozygous for the c.1066-6T>G variant displayed loss of exon 11 in 47%-57% of *ATM* transcripts. Likewise, there is a discrepancy in pathogenicity of the c.1066-6T>G variant with respect to AT. Despite the high population frequency, this variant has been identified only in one patient with AT who was homozygous for this gene alteration. However, this patient exhibited all typical symptoms of classical AT and quantitative analysis performed in lymphoblastoid cell line established from this patient detected the loss of exon 11 in 93% of *ATM* transcripts [28].

Table 2. ATM mutations identified in high-risk breast cancer patients

Family No	**Mutation ∗**	**Predicted effect**	**Diagnosis(age)**	**Cancers in family (age at onset)**
410	c.5177+1G>A	Exon 36 skipped, ter 1680	Bilateral breast cancer (42, 44)	Pancreatic (75), breast (64), breast (?)
116	c.5932G>T	E1978X	Bilateral breast cancer (53,60), ovarian cancer (61)	Ovarian (57), leukemia (?), kidney (?), lung (59)
96	c.6096-9delTTCTT	Exon 44 skipped, ter 2032	Breast cancer (37)	Pancreatic (73)
69	c.1066-6T>G	Exon 11 skipped, ter 372	Breast cancer (32)	Breast (60), breast (?)

∗ *Position in cDNA is according to GenBank accession no. U33841; +1 corresponds to the A of the ATG translation initiation codon*

Bilateral breast cancer and a strong family history of cancer occurred in two patients with the c.5177+1G>A and c.5932G>T truncating mutations. Other patient carrying the c.6096-9delTTCTT alteration was diagnosed with breast cancer before the age of 40. The patient with the c.1066-6T>G mutation developed breast cancer at the age of 32 and two other

members of the family were also affected with this malignancy (Table 2). DNA samples for genotyping were available only in one affected person in each family.

Cells from obligate AT heterozygotes display increased chromosomal instability and have been shown to exhibit elevated radiosensitivity, which suggests that haploinsufficiency at the *ATM* locus may promote tumorigenesis [49,50]. On the contrary, high frequency (~40%) of allelic losses at the *ATM* region in sporadic breast tumors rather supports the classical Knudson's two-hit hypothesis [34]. Nevertheless, the process of inactivation of the retained allele remains poorly characterized. The paraffin-embedded tumor samples were obtained from 3 *ATM* heterozygotes. Tumor tissue was not available in the patient carrying the c.5177+1G>A mutation. In a carrier heterozygous for the c.1066-6T>G mutation, DNA was isolated from metastasis whereas in other two patients from primary tumors. The *ATM* mutations identified in peripheral blood were confirmed in each of the tumor samples.

Immunohistochemical analysis proved loss of expression of ATM protein in all accessible tumor samples from heterozygous mutation carriers, including the sample heterozygous for c.1066-6T>G mutation. In a parallel analysis, breast carcinomas from patients without the *ATM* mutation expressed high level of the ATM protein. To analyze the process of gene inactivation more precisely, we examined tumor samples for LOH at the *ATM* locus and for methylation of CpG islands of the *ATM* promoter. However, none of the tumors from heterozygous mutation carriers displayed LOH at this locus. Accordingly, it seems that the loss of the wild type *ATM* allele is not a common event in breast tumorigenesis. Alternative way of gene inactivation includes promoter hypermethylation. However, our analyses detected hypermethylation of *ATM* promoter only in a carrier heterozygous for the c.1066-6T>G mutation. In this tumor samples, MS-MLPA revealed hypermethylation in 21 out of 27 tested CpGCpG dinucleotides of 25 different promoters including *ATM*. Together with the fact that the DNA was isolated from metastasis, we concluded that hypermethylation of *ATM* in this sample is not causal and is probably connected with the advanced disease. Hypermethylation can result from overexpression of *de novo* DNA methyltransferases common in cancer [51,52] leading to genome instability.

4.2 *ATM* and sporadic breast cancer

In our group of 73 sporadic breast carcinomas, we analyzed *ATM* inactivation by loss of heterozygosity, somatic mutation and promoter hypermethylation.

4.2.1 Loss of heterozygosity

For LOH evaluation, three microsatellites with high heterozygosity (80.6-97.6%) in the *ATM* locus were analyzed. Only one sample was not informative (1.4%). LOH in the *ATM* locus was found in 20.8% samples, which is less than in *BRCA1* (22.7%), *BRCA2* (26.7%) and *TP53* (34.8%) loci [37]. Frequency of LOH in *ATM* is considerably higher than 5%, which is thought to be the likelyhood of random allele loss [33]. These results indicate that *ATM* has a role in sporadic breast cancer tumorigenesis. LOH in *ATM* was uniformly distributed among all histological tumor types as well as in tumors of various immunohistochemical characteristics except protein c-ErbB-2 expression. Interestingly, none of tumors carrying LOH in *ATM* expressed c-ErbB-2 protein whereas in our group only 65.8% of tumors were c-ErbB-2 negative. However, the relationship between c-ErbB-2 and ATM proteins is unknown.

The LOH data were further analyzed using TIGR MultipleExperimentViewer v.4.0 software (available on http://www.tigr.org/software/tm4/menu/TM4_Biotechniques_2003.pdf) [53]. The clustering analysis showed that LOH in *BRCA1* and *BRCA2* are associated and together with LOH in *TP53* make up one cluster whereas LOH in *ATM* represents another group. Thus, it seems that *BRCA1, BRCA2* and *TP53* tumor-suppressor genes take part in one tumorogenic pathway whereas *ATM* in different one.

4.2.2 Mutation analysis

We screened the entire *ATM* open reading frame for pathogenic mutations in tumors with LOH in this locus using PTT. No somatic mutation was identified in the group of 15 tumors with *ATM* allelic loss.

Somatic mutations were not detected in *ATM* in sporadic breast cancer so far and thus, similarly to *BRCA1/2* [37], are considered as rare. Nevertheless, low frequency of somatic mutations can be caused by the fact that the vast majority of studies was dealing with mutation analysis in hereditary breast cancer.

4.2.3 Promoter methylation analysis

ATM promoter methylation was detected in none out of 71 analyzed sporadic breast carcinomas (bisulfite sequencing, MS-MLPA). To date, three studies have been dealing with *ATM* methylation. Vo *et al.* identified hypermethylation of 78% of 23 breast cancer cell lines [41]. On the other hand, neither Treilleux *et al.* [42] (74 sporadic breast carcinomas) nor Allinen *et al.* [43] (30 tumors) have found *ATM* promoter methylation.

We also analyzed promoter methylation in other tumor-suppressor genes by MS-MLPA. With respect to breast cancer predisposition genes, *BRCA1* promoter was methylated in 3 samples and *TP53* in 2 cases. Similarly to *ATM, BRCA2* methylation was not detected in any tested tumor. These results show that hypermethylation of genes involved in pathogenesis of hereditary breast cancer is in sporadic cancer rather uncommon.

Concerning other analyzed genes, the most frequently methylated promoters were *CDKN2B* (cyclin-dependent kinase inhibitor 2B) (89%), *WT1* (Wilms tumor 1) (79%), *PAX5* (69%) and *RASSF1* (Ras-associated domain family 1) (2 probes, 69 and 70%). *CDKN2B* codes for p15 protein which inhibits G1 phase of cell cycle through inhibition of cyclin dependent kinases Cdk4 and/or Cdk6. Methylation of this gene has been described once in 2% (2/105) of sporadic breast carcinomas [54]. However, we detected considerably higher proportion of samples with this gene´s promoter methylation (89%). *RASSF1* tumor-suppressor gene encodes a protein, related to Ras protein, inhibiting cyclin D1 accumulation, which, in turn, leads to cell cycle arrest. *RASSF1* methylation is a frequent event in cancers, particulary in early stage of carcinomas development [55,56]. *WT1* codes for a transcription factor acting as either activator or repressor of many genes. Depending on the rate between expressed isoforms, the breast malignant phenotype can be either enhanced or suppressed [57]. Similarly, *PAX5* also encodes a transcription factor which plays a role in cell differentiation and embryonic development [58]. *WT1* hypermethylation has not been identified so far whereas *PAX5* hypermethylation was detected in approximately 65% of breast carcinomas, which is consistent with our findings [58].

Generally, the samples were methylated in 7 tested dinucleotides CpGCpG on average (median = 7, arithmetic mean = 7,43). Currently, it is not known methylation of which CpG dinucleotides of particular genes leads to significant decrease or eventually to complete transcription silencing. Therefore, this study will be followed by expression analysis of genes with aberrant promoter methylation by real-time PCR.

4.2.4 Immunohistochemistry

The ATM protein was immunohistochemically analyzed in all available samples (46 sporadic breast tumor tissues). ATM expression was decreased in almost all tested samples. However, the lower expression was not associated with LOH.

4.2.5 The ATM gene inactivation

In our group of sporadic breast carcinomas, we detected LOH in the *ATM* locus in 20,8% cases. In addition, neither somatic mutation nor promoter hypermethylation was detected in any analyzed sample. However, the immunohistochemical analysis found

decreased amount of the ATM protein in almost all tumors. Similarly, other studies described lower amount of ATM protein in high proportion of sporadic breast carcinomas (~75%) [42,59]. It was showed that decreased ATM transcription might be caused by lower expression of the DNA-PK protein (a protein kinase which takes part in DSB repair through the mechanism nonhomologous end joining) but a mechanism of such positive regulation is uncertain. Generally according to results of many studies, it can be sum up that expression changes of genes involved in DSB repair are associated with breast cancer pathogenesis [42,59].

5. CONCLUSIONS

Incidence of breast cancer is continuously increasing in the Czech Republic. Tumor development is a result of gene alterations´accumulation, particulary associated with genes involved in regulation of cell growth and division. Hereditary carcinomas account for approximately 5-10% of all breast tumors and in 60-80% cases are caused by a germline mutation in the major predisposition genes *BRCA1* and *BRCA2.* Nevertheless, other genes, mostly of lower penetrance, may play a role in breast pathogenesis such as the *ATM* tumor-suppressor gene.

ATM is the apex of the repair pathway of DSB. This protein kinase activates through phosphorylation of its substrates cell cycle checkpoints, which leads either to the delay of the cell cycle progression until DSB are repaired or to the promotion of apoptosis.

The aim of our study was to establish the *ATM* mutations frequency in high-risk breast cancer patients in our population and specify this gene´s alterations in sporadic breast cancer. That included design and optimalization of method for mutation analysis, loss of heterozygosity detection and methods for detection of *ATM* promoter hypermethylation.

Our results suggest that truncating mutations in *ATM* that occur in AT contribute in heterozygous carriers to the breast cancer development and the splicing variant c.1066-6T>G is not associated with hereditary breast cancer with respect to the occurrence in population. Absence of the ATM protein in all available tumor samples of heterozygous mutation carriers indicates that complete loss of this protein may be required for tumorigenesis. However, the precise mechanism of the second allele inactivation remains obscure.

Concerning the role of *ATM* in sporadic breast tumorigenesis, we analyzed loss of heterozygosity, promoter methylation and somatic mutations in a group of sporadic breast carcinomas. High frequency of LOH indicates that *ATM* is involved in nonhereditary breast carcinomas development. However, we found neither promoter methylation nor somatic mutation in any tumor sample.

To sum up, the *ATM* gene seems to have a role in breast cancer development in a minority of the high-risk families in our population which is significantly lower compared to *BRCA1/2* and it also seems to be involved in pathogenesis of sporadic breast cancer. Despite the *ATM* gene´s length, we do not perform the preventive screening of this gene in breast cancer high-risk families. Nevertheless, we offer the molecular diagnostics of *ATM* to ataxia telangiectasia patients.

6. AKNOWLEDGEMENTS

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