ANALYSIS OF D1853N ATM POLYMORPHISM IN RADIOSENSITIVE PATIENTS WITH CERVICAL CARCINOMA

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Summary: Clinical oncologists have been focusing their efforts on attempting to define risk groups of patients with unusual biological reactions to the recommended therapy regimens using molecular biology techniques. The aims of our study were: (i) to find a design and validate a method for fast and reliable analysis of the D1853N (5557G>A) genetic polymorphism in the ATM (ataxia-telangiectasia mutated) gene; (ii) to use side-directed mutagenesis to generate ATM 5557A-positive DNA (reference ATM5557A DNA); and (iii) to analyze a group of patients suffering from cervical carcinoma with adverse responses to radiotherapy. The 5557A variant was found in three of twenty women (15%). Our data show that the prevalence of the 5557A allelic variant in cervical cancer subjects with adverse responses after irradiation probably does not differ from the prevalence common in Caucasians. A larger population study should confirm these preliminary results.

Key words: ATM; Mutation; Genotyping; Oncology; Diagnostics; Treatment; DNA; PCR; RFLP

Introduction

Current treatment of human malignancies is based in many cases on a combination of radiotherapy and chemotherapy. Adverse response appearing during the cure impairs the clinical state of patients. Side effects of radiotherapy are partially dependent on genetic differences among individuals. Current efforts of clinical oncologists have focused on defining risk groups of patients with unusual biological reactions to the recommended therapy regimens using molecular biology techniques. Several radiosensitivity candidate genes, e.g. ATM, TGFβ1, XRCC1, XRCC3, SOD2, and hHR21, have been identified.

The ataxia-telangiectasia mutated (ATM) gene was localized on the long arm of chromosome 11 (11q22–q23) in 1991 (12). This gene contains 66 exons and its length is 150 kb. The coding sequence is composed of 9168 nucleotides and the encoded ATM protein kinase has a molecular weight of 350 kDa (19). The ATM kinase, a protein with homology to phosphatidyl-inositol-3´ kinases at the carboxyl-terminal part, participates in DNA repair, cell-cycle checkpoints regulation, and apoptosis (27). Irradiation induces ATM serine 1981 autophosphorylation, which initiates appropriate signalling cascades in the cell (22).

A number of missense and protein truncation mutations in the ATM gene were observed in somatic cells of ataxia-telangiectasia patients leading to insufficient repairs of double strand breaks and genetic instability (21, 25). Heterozygous carriers of ATM mutations are clinically unaffected but predisposed to epithelial cancers, especially breast cancer (7, 24). ATM heterozygosity higher than expected in the general population (1–2%) was described in patients suffering from breast, colon, stomach, pancreas, lung, head, neck, uterus, ovarian, endocrine, and skin tumors, T-cell prolymphocytic leukaemia, and B-cell chronic lymphocytic leukaemia (1, 3, 4, 6, 10, 16, 17, 26, 29–31). Thus, ATM mutations are considered a risk factor for carcinogenesis.

Tens of neutral genetic variants and polymorphisms of unknown biological significance in ataxia-telangiectasia (AT) families have also been found (20). The D1853N genetic polymorphism (rs1801516; 5557G>A) in ATM exon 39 leads to the substitution of asparagine for aspartic acid at amino acid position 1853. Previously published papers have demonstrated that the presence of the 5557A allelic variant could enhance radiosensitivity of the carriers, and significantly increases the incidence of adverse responses to radiotherapy of prostate and breast cancer (1, 8).

The aims of our study were: (i) to find a design and validate a molecular biology method for fast and reliable analysis of the ATM gene; (ii) to use side-directed mutagenesis to generate ATM 5557A-positive DNA (reference ATM5557A DNA); and (iii) to analyse a group of women suffering from cervical carcinoma with adverse responses to radiotherapy.

Material and Methods

Clinical samples. Twenty patients of Caucasian origin presented with FIGO stage IIB–IIIB epidermoid cervical carcinoma. The median age was 48 years (range 29–61). Nineteen patients were treated with primary concomitant chemoradiotherapy with doses of 48–50 Gy to the small pel-
visor, 16 of them also received a parametrial boost of 9–14 Gy. In addition, in 13 patients the paraaortic lymphatic nodes were irradiated at 44 Gy. Brachytherapy consisted of 6 fractions with single doses of 4 Gy to point A. Cisplatin with a weekly dosage of 40 mg/m² was administered concomitantly. In one patient, postoperative radiotherapy with a dose of 60 Gy to the small pelvis was applied following radical hysterectomy. With a median follow-up of 5 years (range 2–9), no patients showed any evidence of the tumour. Low gastrointestinal late complications of grade 3 are present in 4 patients, while grade 3 urological toxicity was observed in 4 patients. The genomic DNA of all the patients was extracted from 200 µl of venous EDTA blood by the microcolumn procedure (QIAamp Blood Mini Kit, Qiagen, Germany) with the patients’ informed written consent.

**Preparation of control samples.** DNA of a healthy volunteer was extracted from 200 µl of venous blood as described above. The absence of 5557G>A alleles was confirmed by DNA sequencing. Consequently, target mutagenesis in the codon 1853 was performed. This three-step process included two independent amplification reactions (PCR I and PCR II) inserting adenine instead of guanine into the coding strain, and thymine into the non-coding strain. The last PCR (PCR III) linked both the semi-products together as illustrated in Fig. 1. All the reactions were carried out in 25 µl of reaction mixtures containing 100 ng of DNA (or 5 µl PCR I and II products in PCR III), 10× concentrated PCR buffer with 15 mM magnesium chloride, 200 µM each of dNTP (Takara, Japan), 0.4 µM of the appropriate pair of primers (Generi Biotech, Czech Republic), and 1U of Taq polymerase (Takara, Japan). After initial denaturation (5 min at 95 °C), PCRs were run for 35 cycles consisting of 30 s denaturation at 95 °C, 30 s annealing at 50 °C, and 30 s elongation at 72 °C. The amplification was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems, USA). PCR I amplified the left part (117 bp; forward outer and reverse inner primers were used; the sequences of the primers are provided in Table 1) and PCR II amplified the right part (190 bp; reverse outer and forward inner primers) of the final product. The whole product (286 bp) was formed using outer forward and reverse primers in PCR III (see Fig. 1). The nucleotide sequence of the final product was confirmed by DNA sequencing.

**PCR-restriction fragment length polymorphism analysis (PCR-RFLP).** The temperature profile and the composition of the reaction mixture for PCR were similar to those above, except for the primer sequences. The reverse primer (5´- ATT TCT CCA TGA TTC ATT TGG AT -3´) creating a wild-type-allele-specific recognition site for the restriction enzyme (a mismatched base underlined) by PCR amplification has been previously published (14). The forward primer (5´- AAA CTA TTT GGT GGA TTT GTT -3´) was proposed using Primer Express Version 2.0 software (Applied Biosystems). 10 µl of the product was digested with 10 U of Mbo I restriction enzyme (New England Biolabs, USA) at 37 °C for 16 hours. Digested fragments were electrophoresed on a 3% agarose gel. The PCR product (117 bp) was cleaved with the enzyme into two fragments (95 bp and 22 bp) in the case of the wild-type allele. In all samples containing undigested PCR products visible on the gel, DNA sequencing followed. A mixture without any DNA template served as negative amplification control.

**DNA sequencing.** PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and their nucleotide sequences in both directions were determined with BigDye Terminator v3.1 Cycle Sequencing Kit in Genetic Analyser 3130 (Applied Biosystems) according to the manufacturer’s instructions.

**Tab. 1:** Sequences of outer and inner primers used for target mutagenesis in *ATM* gene exon 39. Underlined bases have created the 5557G>A nucleotide exchange on both strands of PCR products.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5´–3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ATM</em> outer-forward (19)</td>
<td>CATTTCATCATTCCAATATGG</td>
</tr>
<tr>
<td><em>ATM</em> outer-reverse (19)</td>
<td>TCTTAAAATCCATITTTCCTCTTA</td>
</tr>
<tr>
<td><em>ATM</em> inner-forward</td>
<td>GATATTTTACTTCCAATATACA</td>
</tr>
<tr>
<td><em>ATM</em> inner-reverse</td>
<td>TGGATTTGGAGTAAATATAC</td>
</tr>
</tbody>
</table>

**Fig. 1:** A design of target mutagenesis in *ATM* exon 39. Part A shows annealing sites of all used outer and inner primers. Part B shows PCR products generated during three-step target mutagenesis. M means the target nucleotide exchange (5557G>A).
Results and Discussion

ATM kinase has a very important role in the maintenance of genome integrity. The ATM gene belongs to conserved parts of human DNA. Table 2 summarizes a sequence similarity of ATM homologues of several animal species and humans in the ATM region covering the human codon 1853. The frequency of the ATM 5557A allelic variant in AT patients is about 18% (20). A calculated prevalence of 5557A homozygotes is 3% and predicted heterozygosity in AT patients is about 30%. Previously published studies showed that not only AT patients but 15–26% of the Caucasian population as well carry the 5557A allele. 1–2% of Caucasians are thought to be homozygotes with clinical symptoms of ataxia-telangiectasia (3, 11, 17, 19). The Finnish population reveals a heterozygosity of 37% and homozygosity of 5% (28). A low distribution of the 5557A, on the other hand, was described in African-American, Latino, and Japanese population groups (8%, 13–14% and 6%, respectively) (5, 14). The mentioned inter-ethnic discrepancy provides evidence for the geographical heterogeneity of the 5557A allele in the human population.

For DNA analysis, we used a PCR-RFLP method. This approach (if used for a limited number of polymorphic sites in the gene) enables the analysis of more clinical samples per day, cheaper examination, and simplifies the evaluation of the results in comparison with direct DNA sequencing. Firstly, the pair of amplification primers and Dde I restriction enzyme according to Maillet et al. (19) were attempted. Despite the fact that the primers appeared in other papers as well (3, 18), we received false negative results of our analyses. Therefore, we have defined our own combination of PCR primers. The reverse primer creating a recognition site for Mbo I restriction enzyme was designed previously. However, the authors used Rs a I enzyme with a different recognition site than Mbo I (14). In all cases, our PCR-RFLP gave completely concordant results with DNA sequencing. Fig. 2 demonstrates the electrophoretic mobility of restriction fragments after Mbo I digestion. Fig. 3 provides an example of DNA sequencing.

Fig. 2: A slab gel electrophoresis on 3% agarose gel. Line 1: DNA marker 25 bp (New England BioLabs); line 2: positive control with 5557G>A mutation in the homozygous state; lines 3, 5 and 6: wild-type patients; line 4: heterozygote patient; line 7: negative control without DNA template. The samples in the lines 2, 3, and 4 were sequenced (Fig. 3).

<table>
<thead>
<tr>
<th>Source</th>
<th>Location (chromosome)</th>
<th>NCBI Accessions</th>
<th>DNA Sequence Homology * (5′→3′)</th>
<th>Human Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>11</td>
<td>NP_000042.3</td>
<td>att tta ctc caa gat aca aat gaa tca</td>
<td>99,6</td>
</tr>
<tr>
<td>Pan troglodytes (chimpanzee)</td>
<td>11</td>
<td>XM_001138999.1</td>
<td>att tta ctc gat aca aat gaa tca</td>
<td>99,4</td>
</tr>
<tr>
<td>Bos taurus (cow)</td>
<td>15</td>
<td>XM_002692978.1</td>
<td>att ctg caa gat aca gat gaa tca</td>
<td>90,7</td>
</tr>
<tr>
<td>Canis lupus familiaris (dog)</td>
<td>5</td>
<td>NM_001130828.1</td>
<td>att tca ctg gaa gac aat gaa tcg</td>
<td>89,0</td>
</tr>
<tr>
<td>Mus Musculus (mouse)</td>
<td>9</td>
<td>NM_007499.2</td>
<td>gtt tta ctg gaa gat aca gat gaa tcc</td>
<td>85,0</td>
</tr>
<tr>
<td>Rattus norvegicus (rat)</td>
<td>8</td>
<td>NM_001106821.1</td>
<td>gtt tta ctc gaa gat aca gat gaa tcc</td>
<td>84,1</td>
</tr>
<tr>
<td>Gallus gallus (chicken)</td>
<td>1</td>
<td>NM_001162400.1</td>
<td>act gac att agt gaa aat gag tct</td>
<td>not analysed</td>
</tr>
</tbody>
</table>

* Human ATM codon 1853 in bold; nucleotide discrepancy to human DNA in the region around the codon 1853 changing amino acid sequence underlined

Tab. 2: Human ATM gene homology according to the National Center for Biotechnology Information (NCBI), USA.
No homozygote was found. It seems the prevalence of the 5557A heterozygosity was identified in three of twenty cases of women with cervical carcinoma after radiotherapy. The prevalence of the 5557A allele in a small group of radiosensitive adverse reactions (8). In our study we analysed the predispose prostate cancer patients receiving radiotherapy to possession of one altered copy of the MS gene. Several studies have also shown a positive association between the 5557G allele and 3,780 controls reported that the 5557G>A polymorphism could not associate with a higher risk of breast cancer and 3,780 controls reported that the 5557G>A polymorphism cannot associate with a higher risk of breast cancer development (13).

Several papers also found a positive association between the 5557G allele in a homozygous state and enhanced clinical radiosensitivity. Angele et al. described a higher frequency of the 5557A homozygotes (6%) in radiotherapy-sensitive breast cancer cases (3). Andreassen et al. found five heterozygous (12%) and two homozygous (5%) 5557A carriers in a group of 41 breast cancer patients with radiation-induced subcutaneous fibrosis (1). Cesaretti et al. observed that the possession of one altered copy of the ATM gene may predispose prostate cancer patients receiving radiotherapy to adverse reactions (8). In our study we analysed the prevalence of the 5557A allele in a small group of radiosensitive women with cervical carcinoma after radiotherapy. The 5557A heterozygosity was identified in three of twenty cases (15%). No homozygote was found. It seems the prevalence of the 5557A allelic variant in cervical cancer subjects with adverse responses after irradiation does not differ from the common population prevalence determined in Caucasians. However, an exact mathematical analysis was not possible because of the low number of probands at this moment, so our preliminary results need confirmation in a larger population of patients.

The published data indicates that the presence of 5557A could not be a sufficient genetic event influencing the optimal dosage of radiotherapy. Other mutations in the ATM gene, e.g. 146C>G, 2119T>C, IVS10-6T>G, 7271T>G, IVS62+60G>A, IVS24-9delT, and IVS38-8T>C have been observed to associate with breast, lung, prostate, oral cavity, pharynx, and skin cancer in some but not all published studies (2, 10, 11, 14, 16, 23). A wider list of genetic changes occurring in the ATM gene also includes: 378T>A, 735C>T, 1066-6T>G, 1810C>T, 2572T>C, 3161C>G, 4258C>T, 4578T>C, 5071A>C, 5558A>T, 7390T>C, IVS62+8A>C, and many other mutations. In summary, we speculate that there is no unique region in the ATM gene or a single causative allele discriminating the carriers for earlier growth of tumours, or indicating the necessity of an individual approach to radiotherapy.

Taking into account a multistep process of carcinogenesis, some authors tried to define the importance of the ATM kinase in double-strand break DNA repair and the number of genetic changes observed in the ATM gene as “risk ATM haplotypes” with a predisposition for cancer development. The 5557A allele was found to be in strong linkage disequilibrium with the IVS38-8T>C variant (in cis position) in breast and prostate cancer (2, 14, 15). Other authors, however, did not support any association between these mutations and breast cancer (28). Kim et al. described the role of specific haplotypes in the ATM gene in the development of lung cancer in the Korean population (16). Angele et al. reported that the specific ATM haplotype containing the rare alleles IVS22-77T>C and IVS48+238C>G is more frequent in breast cancer, especially in homozygote combination (3). The same papers have shown that the presence of IVS22-77T>C/IVS48+238C>G composed heterozygosity in breast cancer could be associated with a radio-protective effect against fibrosis in normal tissue after irradiation. We believe these observations should clearly reveal how ATM polymorphisms influence abnormal radiosensitivity in the patients.

Several studies have also demonstrated possible functional relationships between ATM variants and polymorphic sites in other genes, especially in APEX1, hHR21, OGG1, POLB, RAD54L, RECO1, TGF1, SOD2, XPF, XRCC1, XRCC2, and XRCC3 (1, 17). Cortez et al. presented functional interactions between ATM and BRCA1 proteins (9). Maillet et al. found the association of the 5557G>A polymorphism with a higher incidence of colorectal cancer in MLH1 or MSH2 germ-line mutation carriers mentioned above (18). All these “radiosensitivity candidate genes” manifest the complexity of genetic events appearing in the

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Fig. 3: DNA sequence analyses of ATM exon 39. The upper electrophoreogram shows the nucleotide sequence of the mutant control sample (5557A allele); the middle part presents the homozygous sequence of a wild-type patient (5557G allele); and the lower part shows the sequence of a heterozygous 5557G>A patient. Arrows indicate the site of mutation.
process of carcinogenesis and the underlying appearance of adverse tissue reactions after radiotherapy.

Conclusions

In our study we validated the PCR-RFLP method using the Mbo I restriction enzyme. Positive control samples carrying the homozygote 5557A genotype in the ATM gene were successfully constructed. Three of the twenty women (15%) in our study suffering from cervical carcinoma with adverse responses to radiotherapy were heterozygotes for the 5557A allelic variant. Thus, our preliminary data show that the prevalence of the 5557A allelic variant in cervical cancer subjects with adverse responses after irradiation probably does not differ from the prevalence common in Caucasians. A larger population study should confirm the preliminary results.

Acknowledgement

The study was supported by research projects NT11334-4/2010 and MZO 00179906 from the Ministry of Health, Czech Republic.

List of abbreviations

5557A allelic variant of the ATM gene
5557G wild-type allele of the ATM gene
AT ataxia-telangiectasia
bp base pairs
D1853N substitution of asparagine for aspartic acid at position 1853
dDe I Desulfovibrio desulfuricans
dNTP deoxynucleotide triphosphate
Gy Gray; unit of irradiation
HNPPC hereditary non-polyposis colon cancer
kb kilobases
kDa kilodaltons (molecular weight)
Mbo I Moraxella bovis
NCBI National Center for Biotechnology Information
(USA)
PCR polymerase chain reaction
RFLP restriction fragment length polymorphism
Rsa I Rhodobacter sphe roaerides
APEXI, ATM, BRCA1, hHR21, MLH1, MSH2, OGG1, POLB, RAD54L, RECQL, SOD2, TGFBI, XPF, XRC1, XRC2, and XRCC3 are official names of genes appearing in the Online Mendelian Inheritance in Man (OMIM), Johns Hopkins University and on pages of the HUGO Gene Nomenclature Committee (http://www.genenames.org/).


References


Received: 19/10/2010
Accepted in revised form: 25/04/2011

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