Five-hit hypothesis in ATM gene: An individualized model in a breast cancer patient

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1. ABSTRACT

   The aim of this study was to trace D1853N in a proband affected with primary BC to explore the molecular, cellular and structural aspects of ATM. Exon 37 and splicing regions were PCR-sequenced. Allelic location of the alterations was determined by molecular cloning. Possible impact of alterations was investigated through the bioinformatics and protein expression assays. Five genetic variants including IVS 36-91 AA>TT, IVS 36-8 T>C, D1853N, IVS 37+47 A>G, IVS 37+60 Del T were found in the target regions of ATM and all the alterations were occurred heterozygously. IVS 36-8 T>C and D1853N were observed in blood and tumor tissue, whilst splicing variants were only occurred in tumor tissue. Missense D1853N alteration seems to be effective on 2D and 3D structure of ATM protein and the probability of splicing found to be decreased by intronic variants. Protein expression of ATM also confirmed the occurrence and functional impact of alterations. Results reflect a five-hit hypothesis in a proband with BC that influence ATM, as a guard of genomic stability, at molecular, cellular, and structural levels.

2. INTRODUCTION

   As a major public health problem, breast cancer (BC) is the most frequent cancer in women with 1.4 million new cases and approximately 459,000 deaths globally. In spite of advances in technology and diagnosis approaches in cancer, increase in incidence and related death has been observed by 18 percent from 2008 cases according to GLOBOCAN statistics (1). As similar as the most cancers, BC has mostly been observed sporadically with nearly frequency of 90-95 percent, whilst 5-10 percent of BC is occurred as inherited type (2). The most reported genetic loci involved in BC are reported as four categories including high penetrance (BRCA1, BRCA2, and P53), intermediate penetrance (ATM, CHECK2, BRIP1, and PALB2), low penetrance (some SNPs), and uncertain penetrance (PTEN, STK11, CDH1) (2, 3).

   ATM gene is located at chromosome 11q22.3 that encodes an instruction for producing a 370-kDa-protein, as a member of the phosphatidylinositol 3-kinase (PI-3 kinase) family of Ser/Thr protein kinases. This protein is an important cell cycle
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checkpoint kinase and regulates different proteins including P53, BRCA1, CHK2, RAD17, RAD9, and NBS1. It plays a crucial cell response to DNA damage and consequently genome stability (4-6). To date, more than 600 mutations have been reported in ATM gene in different mutation databases. In addition to ataxia telangiectasia, mutation of ATM has also been reported in cancers including breast, stomach, bladder, pancreas, lung, and ovarian (7, 8). D1853N is one of the alterations that has previously been found to be more frequent in BC patients compared to the control groups (9-11). Also, we have previously reported that D1853N polymorphism along with IVS 38-63T>A and IVS38-30A>G formed an important triangle in exon 37 of ATM gene and its 5'-splicing region in development of astrocytoma in an Iranian proband with astrocytoma, as three-hit hypothesis (12).

According to our previous data on hit-hypotheses in ATM, we aimed to explore the exon 37 of ATM by considering the D1853N polymorphism, as a predisposition factor, and possibly the additional evolutionary alterations at genomic and somatic level in a proband affected with primary BC.

3. MATERIALS AND METHODS

3.1. Design of study

This study is designed on the basis of: 1) The multi-disciplinary insight by tracing the origin of the D1853N polymorphism in the pedigree of patient, 2) The remarkable early age of proband’s onset, 3) Performance of molecular investigation at genomic and somatic levels, and 4) Performance the functional protein expression assay as the complementary study at cellular level.

3.2. Proband information and clinical data

Based on the genetic counselling, a female proband without any family history (FH) of cancers in her pedigree, aged 34 years old was studied as a referral patient to the unit of cancer Genetics, Tehran University of Medical Sciences, Tehran, Iran. She was affected with an invasive ductal carcinoma of the left breast, the tumor of breast was diagnosed as a grade of 3, size of 3 centimeters, and positive estrogen receptor. Regarding the metastasis, vascular- and peroneural invasion were reported. In addition, 5 out of 9 auxiliary lymph nodes revealed to be metastatic. This study was approved by the Ethic committee of Tehran University of Medical Science (Tehran, Iran) and the patient has signed the consent form.

3.3. DNA extraction, Polymerase chain reaction (PCR), and sequencing

Total DNA extraction from blood and tumor tissue of proband was performed using two different methods including salting out and phenol-chloroform, respectively. Quality and quantity of extracted DNA were checked by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) and the integrity of DNA was assessed using 1 percent agarose gel electrophoresis. Then, the target region of ATM was PCR-amplified according to the following program: initial denaturation at 95°C for 4 min, 32 cycles of denaturation at 95°C for 35 s, annealing for 32 s at 53.5°C, extension at 72°C for 36 s and a final extension at 72°C for 5 min. Specific used primers were Forward (5’ TTTAATATGTCAACGGGGCAT -3’), Revers (5’ CCATCTTTAAATCCATCTTTCTC -3’) that produced an amplicon with size of 342 bp. PCR products were purified using PCR Cleanup Kit (Roche, Germany) and sent to be sequenced.

3.4. Molecular cloning

Based on TA cloning method in our previous report (13), purified PCR products containing 3'-AA overhang were ligated into PTZ57R (T) vector using InstAclone PCR Cloning Kit (Fermentas, Lithuania) according to manufacturer’s instruction. E. coli DH5alpha was then transformed with 3 μl of ligated vector. Recombinant colonies were determined using LB agar medium containing IPTG/X-gal and ampicillin. Mini-prep of recombinants was performed in LB broth medium. Finally, plasmid extraction was performed using EasyPure HiPure Plasmid MiniPrep Kit (Transgen, China) and extracted plasmid samples were sequenced using M13 universal primer.

3.5. Immunofluorescence (IF) assay

The cell extraction, and conjugation processes were performed according to our previous publications (12, 14). Two profiles were assayed by IF: Profile A: Monoclonal mouse anti-human p53, conjugated with FITC, 2. Monoclonal mouse anti-ATM antibody, with Rpe and 3. Monoclonal anti-p63 antibody, detected with Pe-cy5. The corresponding mouse immunoglobulin was used as negative control for the same population of this triple profile. Profile B: Monoclonal cyclin E conjugated with FITC, Monoclonal cyclin D1 conjugated with Rpe, and monoclonal ATM antibody conjugated with Rpe-cy5 were used. The corresponding mouse immunoglobulin was used as negative control for the same population of each triple profile.

3.6. In silico analysis

The In silico analysis of coding variations was accomplished by SIFT, PolyPhen, MUpro, and Project HOPE. Impact of coding alterations on two and three dimensional structure (2D and 3D) of target region of ATM protein was evaluated using PSIPRED, PHD, and Jpred 4 (for secondary structure prediction), I-TASSER, VADAR, Galaxy refine, TM-align and SuperPose (for tertiary structure prediction). On the
other hand, effect of splicing alterations was predicted by NetGen2 software from CBS prediction services and Human Splice Finder (HSF) system.

4. RESULTS

4.1. Sequencing findings

As it is shown in Figure 1, two genetic alterations including a splicing variant of IVS 36-8 T>C and missense alteration of D1853N were found in blood tissue of proband in homozygous status. D1853N was traced in pedigree and was found to be inherited from proband’s mother. Although IVS 36-8 T>C was observed in both blood and tumor tissues, it was not inherited. In addition to mentioned hits, three intronic hits of IVS 36-91 AA>TT, IVS 37+47 A>G, and IVS 37+60 del T were only found in tumor tissue of proband and were not inherited.

4.2. Allelic location of hits

After determining of alterations based on sequencing findings, allelic location of each of hits was discovered by cloning-sequencing. As it is illustrated in Figure 2 a-e, sequencing of different clones showed that IVS 36-91 AA>TT occurred in heterozygous and present on both the same and different allele of IVS 36-8 T>C and D1853N. Also, IVS 37+47 A>G and IVS 37+60 Del T were found to be present in heterozygous on the same allele that INV 36-8 T>C and D1853N were occurred but in different allele in which IVS 36-91 AA>TT was located. IVS 36-8 T>C and D1853N were also occurred in heterozygous and presented on the same allele.

4.3. Immunofluorescence (IF)

The results of protein expression (PE) assay is provided by two different triangle profiles: Profile A: The provided image presents the PE of P53 and ATM and P63 with a harmonic expression and co-expression (Figure 3 a-f). Profile B. presents the protein expression of Cyclin D1, Cyclin E, and ATM (Figure 4 a-f). Cyclin E and cyclin D1 are revealed to have a heterogenic pattern of PE with majority of cells with high expression. Besides, ATM reflects lack of PE in majority of BC tumor cells.

4.4. In silico predictions

In silico analysis of D1853N coding variation indicated that this hit was probably damaging. 2D and 3D structure modeling were performed for exon 37 of ATM. D1853N was the only coding alteration that disclosed to be effective on 2D and 3D structure of target region (Figure 5). Prediction of 2D structure using mentioned tools showed that this missense alteration introduces some structural changes through disturbing the percentage and size of shits and coils (Figure 5). Alignment of 3D structure of wild type and mutant type was also revealed that D1853N creates some structural difference (Figure 5). Prediction of intronic variants by NetGene2 and HSF showed IVS 36-91 AA>TT had no impact on splicing, IVS 38-8 T>C decreased the probability of main splice site from 33 to 26 percent, IVS 37+47 A>G decreased the probability of main splice site from 33 to 31 percent, and IVS 37+60 Del T led to deletion of the acceptor splice sites in complement strand.

5. DISCUSSION

Encoded protein by ATM is involved in control of the rate of cells grow and division, normal development and activity of nervous and immune system. However, the milestone of ATM activity is response of cells to damaged or broken DNA so that mutation and dysfunction of ATM can lead to cancer
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It is reported that carriers of mutation in ATM have 2-fold higher risk for BC. Association and implication of ATM common variants in predisposing to BC have been studied but results had been inconsistent (16, 17). The role of D1853N polymorphism as a predisposing factor has been previously reported in BC patient (9-11, 17). In the current study D1853N was traced in a proband with BC to explore the molecular, cellular and structural aspects of ATM. Our results revealed a five-hit hypothesis in ATM exon 37 and its surrounding splicing sites. In silico and expression assays also confirmed the occurrence and possible pathogenic effects of alterations.

The five hits included IVS 36-91 AA>TT (c.5897-91 AA>TT), IVS 36-8 T>C (c.5897-8 T>C), D1853N, IVS 37+47 A>G (c.5674+47 A>G), and IVS 37+60 Del T (c.5674+60 Del T). IVS 36-91 AA>TT was observed both on the same and different allele in where IVS 36-8 T>C and D1853N are present. IVS 37+47 A>G and IVS 37+60 Del T were only seen on the same allele where IVS 36-8 T>C and D1853N are occurred. An adult human body has been approximately made up of $10^{14}$ cells with same genome but different gene expression and epigenetic signature which have originated from one cell known as zygote. After forming zygote, its division leads to produce of blastomeres from which blastula will be made up. In the process of gastrulation, three major germ layers consisted of endoderm, mesoderm, and ectoderm will arise that will finally build up human body organs. According to different literatures, it is believed that differentiation and specialization of cells are initiated either before or after gastrulation (18-20). D1853N was inherited polymorphism from proband’s mother, however, IVS 36-8 T>C variation was not inherited and observed both in blood and tumor tissue of proband and indicates that it has been occurred before differentiation at the very early stage of embryogenesis.

In addition, further hits sound to be occurred through the course of tumor evolution at somatic level. IVS 36-91 AA>TT, IVS 37+47 A>G and IVS 37+60 del T were three intronic variants observed in 5’ and 3’ splicing sites of exon 37 of ATM (Figure 1). These intronic variations were observed at somatic level in tumor tissue of proband. It is clear that early events at very early stage of differentiation could be vital and could be under the influence of both environmental and genetic predisposing factors presented in key genes. We have previously reported a three-hit hypothesis including D1853N in conjunction of IVS 37+63T>A and IVS 37+30 A>G within ATM gene as a triangle predisposing genetic status in astrocytoma development in an affected proband (12).

In addition to clinical observation indicating of possible role of ATM, as the genome stability guard, in predisposing of individuals and especially the proband affected with an early primary BC to cancer, In silico and expression analysis were also performed to predict the probable pathologic effects of five hit observed in ATM exon 37 and its splicing regions.

According to dbSNP and COSMIC catalogue, D1853N is a coding polymorphism (rs1801516) and considered as a predisposing sequence variant. According to analysis performed by different bioinformatics tools, the residue of interest is located in a disordered region and the protein is predicted to be partially disordered due to D1853N. The mutated residue is located in a domain that is important for the main activity of the protein and mutation of the residue might disturb this function. There is a difference in charge between the wild-type and mutant amino acid and this can cause loss of interactions with other molecules or residues. 2D and 3D structure prediction of ATM exon 37 also disclosed that D1853N exert structural variations by disturbing the percentage of...
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Intronic variants might affect alternative splicing of the mRNA (24). Intronic variants could also be occurred in enhancers, therefore alterations in these regions could influence on expression of many genes (26). We can conclude that variations occurred in invariant 5’-GT and 3’-AG and other conserved splicing sequence may lead to inaccurate splicing, abnormal alternative splicing, and gene dysregulation.

In the current work, IVS 36-91 AA>TT, IVS 38-8 T>C, IVS 37+47 A>G and IVS 37+60 Del T were observed as non-coding intronic variants that could influence adversely the splicing of ATM exon 37. In silico analysis of these variants showed that all the intronic alterations except IVS 36-91 AA>TT have potential consequences.

Figure 3. Mode of Protein expression of P53, ATM, and p63 in the tumor of a patient with breast cancer. a) Breast tumor cells with dapi; b) The same tumor cells conjugated with FITC presenting the expression of p53; c) Same cells with Rpe showing the expression of P53. d) Same tumor cells conjugated with Pe-cy5, reflecting the p63 expression e), Co-expression of P53/ATM/p63; f) Merged image of dapi/ P53/ATM/p63. Magnification of cells: x100.

Figure 4. Protein expression of Cyclin D1, Cyclin E, and ATM in the tumor cells of a breast cancer patient. a) Breast tumor cells with conjugated with of cyclin E1; b) The same tumor cells with Rpe presenting the expression of cyclin D1; c) Same cells with Rpe showing the expression of ATM conjugated with Pe-cy5; d) Co-expression of cyclin E/cyclin D1/ATM; f) Merged image of dapi/ cyclin E/cyclin D1/ATM. Magnification of cells: x100.

After identifying the splicing of immature pre-mRNA, there are several reports that indicate DNA variations adversely affective on normal splicing are related to human genetic diseases (21-23). In contrast to coding variants, splicing-related alterations introduce pathological effects through disrupting of the normal removing of introns and rejoining of exons and therefore disturb the formation of normal and functional mRNA (24). Intronic variants might affect alternative splicing of the mRNA (25). Intron variants could also be occurred in enhancers, therefore alterations in these regions could influence on expression of many genes (26). We can conclude that variations occurred in invariant 5’-GT and 3’-AG and other conserved splicing sequence may lead to inaccurate splicing, abnormal alternative splicing, and gene dysregulation.
to disrupt the accurate splicing by either decreasing the probability of main splice site or deletion of the acceptor splice sites in complement strand. Although the most straightforward and reliable method to detect splicing defects are functional studies and analysis of RNA, the In silico analysis revealed that the splicing variants in ATM might be pathologic for cells and carrier’s proband.

Figure 5. 2D and 3D structure prediction of ATM exon 37 containing D1853N genetic alteration. A. It is obvious that substitution of Aspartic acid in codon 1853 with Asparagine leads to structural changes through disturbing the percentage and size of shits and coils (outlined with rectangle and circles). B. Regions outlined with rectangles are indicating of differences that D1853N convey on ATM protein structure. Also, folding of turns seem to be different between wild (D1853) and mutant (1853N) type.

To confirm the clinical and In silico findings related to adverse effects of molecular alterations in ATM gene, exploration of protein expression is an essential performance. In this regard, two profiles are provided (Figures 3 and 4). In Profile-A, protein expression of p53/ATM/p63 shows co-expression of the protein of three tumor suppressors. (Figure 3). However, there is a single cell in the images provided for ATM
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and p63 (Figure 3 c,d) and not in p53 (Figure 3 b). This single cell may be facilitating to form a clone of cells with normal functional capacity which is a positive benefit for our patient affected with breast cancer. Besides, this single cell may be considered as a sign for good prognosis as well.

As profile- B shows, cyclin E and cyclin D1 both are indicative of a heterogenic expression including high and low modes, but cyclin E has higher PE than cyclin D1. However, the impact of these two genes on proliferation, progression and malignant statue in the BC patient is remarkable (Figure 4 a,b). Furthermore, very few cells have high PE for ATM gene which is a negative sign for the patient. So, besides the sequential molecular hits through the patients' life, estimation of protein expression as the final step of molecular alteration may pave the way to plan for an early detection and the most influential clinical management (s) and possibly therapeutic innovation.

In conclusion, the cascade of molecular events led to the functional diversity in these key cell cycle related tumor suppressor genes. These step by step events are indicative of the evolutionary processes during the patient's life, including at pre-, post-embryonic and post-birth epoch. These scenario is reflective of the remarkable cooperation between genetic and tumor biology which are two end points of a link. The message of the current results highlights the importance of personalized insight in cancer managements including prediction, prevention and prognostic implications, not even for the probands, but also for their relatives through the pedigrees.

The In silico prediction approaches are speed, flexible, low cost and less time-consuming efforts in comparison to in vitro and in vivo methods that is why they have gotten attention in the last few decades in various types of research. However, none of bioinformatics tools have shown complete accuracy and there sometimes are inconsistency in predicted results. Furthermore, as all features and criteria are not considered in the In silico prediction approaches, there may be a discordance between In silico predictions and in vitro or in vivo functional studies. Therefore, functional assay is generally the best and reliable tool to characterize the biological effect of variants, for example, the patient's RNA analyses by RT-PCR to verify located in intronic regions or assessing of protein expression to determine the impact of variants on the expression level of interested gene. In conclusion, bioinformatics can be used to filter more likely disease-causing variants from among the large number of candidates and In silico predictions can subsequently be determined, verified and confirmed in vitro and in vivo by functional assays.

6. ACKNOWLEDGMENTS

We would like to thank the patient for her cooperation and providing the required Information. The authors declare that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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**Abbreviations:** BC: Breast cancer; ATM: Ataxia telangiectasia-mutated; Family History (FH); Human Splice Finder (HSF); 2D: Secondary structure; 3D: Tertiary structure; IF: Immunofluorescence; PE: Protein expression;

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