

Eight-Hit Evolutionary Pattern in ATM Gene of a Breast Carcinoma Patient: A Personalized Approach

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Abstract: *Background:* Different genetic variants in the ATM gene have been reported to be associated with breast carcinoma (BC). Purpose of the study was to consider the multi-insights experiments and explorations through the molecular, cellular and structural aspects of ATM.

Methods and Results: D1853N polymorphism was traced in a proband with BC. DNA extracted from blood and tumor tissue was PCR-amplified and cloned to allelic localization of characterized variants. Protein expression and In silico analysis based on three different profiles involved in cell cycle were also performed to confirm mutational events. Findings revealed the molecular based sequential events as an eight-hit evolutionary pattern (8- Hit E.P) including D1853N as the first predisposing hit (inherited), pre-differentiation stage hits including IVS 36-8 T>C as the 2nd hit, V1833M as the 3rd hit, L1888L as the 4th, and somatic variants including IVS 36-46 C>T, L1842L, H1864H, and S1872R were considered as the 5-8th hits. Low protein expression of ATM in the majority of cells was observed, but the expression of cyclin E, CDC25A, P53, and Ki-67 was more diverse.

Conclusions: Observations were reflective of the sequential molecular and cellular events through the entire patient's life from the pre-differentiation embryonic stage and all through the post-birth periods. Mentioned hits seem to be effective on expression and function of ATM which confirmed by the expression and in silico analysis.

Keywords: Ataxia Telangiectasia Mutated gene (ATM), Breast carcinoma, Hit-Evolutionary pattern, Hypothesis, Protein expression, In silico, Personalized management, innovative therapy.

INTRODUCTION

The malignant breast cells are formed through the multi-developmental episodes including the programmed proliferation accompanied by a different cascade of events to guarantee the occurrence of breast carcinoma (BC). BC is reported as the most common malignancy within women in almost all of the geographical regions [1]. A variety of factors including physiological, environmental, and family history (FH) are involved in the development of BC [2]. Several genetic factors have been recognized to be linked with BC susceptibility. However, *BRCA1/BRCA2*, *p53*, *PTEN*, *STK11/LKB1*, *CDH1*, *ATM*, *CHECK2*, *BRIP1*, and *PALB2* genes are highlighted as the most important genetic risk factors in BC patients [1, 3].

Ataxia telangiectasia-mutated (*ATM*) gene is located at chromosome11q22-q23 providing a 13 kb transcript instructions for making a 350 kDa serine/threonine protein kinase. ATM is recruited to the sites of double strand breaks (DSBs) by Mre11-Rad50-Nbs1 (MRN) complex and helps cells recognize damaged or broken DNA through activation of cell

cycle checkpoint elements including P53, NBS1, CHEK2, and BRCA1. Hence, it is known as the first sensing component of DNA damage [4]. The loss of function of ATM directly leads to Ataxia telangiectasia (AT), furthermore, carriers of *ATM* mutations have a higher risk of developing BC. Accordingly, we have reported that the missense substitution of aspartic acid with asparagine in codon 1853 (D1853N) is noticeably more frequent in BC patients than the control groups [5]. Besides, the D1853N could be detected either in healthy individuals or carcinoma patients.

As a micro-evolutionary process, initial hit in the key genes such as oncogenes, tumor suppressors, and repair genes could increase the chance of different mutation acquiring in cells by conferring a growth advantage or by inducing genomic instability [6]. The real Hit introduced by Knudson refers to a sequential/molecular alteration in a specific gene and in its specific exon and intron known as two-hit hypothesis in development of retinoblastoma [7-9]. The multi-hits could occur at different stages including pre-differentiation, through the whole embryonic period, post-birth and through the individual's life. However, a predisposing element, such as D1853N polymorphism in our case is hypothesized to be essential for initiation and promotion of HIT- process [9].

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Our aim was based on; 1) The critical role of D1853N polymorphism; 2) tracing D1853N and possibly the occurrence of evolutionary hits in a BC patient; 3) our previous publications on the importance of D1853N (rs1801516) in the primary BC patients [5], and 4) formulating our three-hit hypothesis [8]. Cooperation, harmonic behavior, restriction, supporting, aggressiveness are examples of the multi-paradigm insights which are rather confusing in carcinoma. As the final aim, combating against carcinoma as a challenging issue requires multi-directional insights. Therefore, design of this project was based on the multi-insight experiments and exploration through the molecular/cellular/structural aspects of *ATM* gene as a frame of triangle support for our findings.

MATERIALS AND METHODS

Patient's Information

A pedigree-based investigation was performed on a female proband, aged 42 years old, affected with infiltrating ductal carcinoma (IDC) of the right breast who was referred to the unit of Cancer Genetics, Tehran University of Medical Sciences. The tumor was characterized with grade III, size of 4.5. Centimeters, and 28/45 auxiliary lymph nodes were revealed to be metastatic. Furthermore, the fibrocystic changes of non-tumoral breast tissues were reported. Core tissue biopsies were provided in size of 2 × 3 × 4cm for downstream analysis. The current study has been approved by the Research Ethic Committee of Tehran University of Medical Science (Tehran, Iran) and a signed informed consent form (according to the Declaration of Helsinki) was taken from probands. The comprehensive pedigree was drawn by the cancer genetic counselor through multi-counselling and face to face interview (S. Figure 1).

Pedigree Analysis

There was no FH of BC in her paternal or maternal sides, but her first son was affected with right BC at age of 41- years old. The FH of other malignancy in the relatives of our proband included second cousin with brain tumor at age of 20 years who deceased few months later, her mother was affected with lymphoma at age of 63 years who deceased at the age of 74, and the proband's far paternal relative with benign uterus neoplasm at the age of 60 and deceased 10 years later (S. Figure 1).

Design of Study

The design of this investigation was based on; 1) An early age of onset of the proband, 2) the pedigree-

based insight to record the confirmed information and documentation of the informative key facts, 3) multi-sampling approach including the peripheral blood and other tissues, and 4) considering the molecular and cell-based strategies. This study has been designed and performed at Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Preparation of DNA, Polymerase Chain Reaction (PCR), and Sequencing

Total DNA was extracted from proband's tumor tissue and her peripheral blood by the phenol/chlorophorm method. The PCR reaction was run in Veriti® Thermal Cycler (Applied Biosystems, USA) according to the previous report [8]. Primer sequences used to amplify the target fragment of *ATM* were Exon-37F (5'- TTTAATATGTCAACGGGGCAT - 3') and Exon-37R (5' CCATCTTAAATCCATCTTTCTC -3'), generating products were of 342 bp. PCR products was directly sequenced (ABI 3130XL Genetic analyzer).

Molecular Cloning

The PCR products were purified using PCR Cleanup Kit (Roche, Germany). Purified PCR products were cloned into PTZ57R (T) vector using InstAclone Kit (Fermentas, Lithuania) according to manufacturer's instruction. Selection of recombinants was performed as previously reported [8-10]. Plasmid extractions were performed from recombinant colonies, extracted plasmids were electrophoresed on 1% agarose gel, and those showing 342 bp larger size than control (vector without insert) were sequenced.

Two-Dimension (2D) and Three-Dimension (3D) Structure Prediction of Mutant *ATM*

Impact of molecular alterations found in proband on secondary (2D) and tertiary (3D) structure of *ATM* protein was analyzed using bioinformatics tools including PSIPRED, PHD, and Jpred 4 (for secondary structure prediction), I-TASSER, VADAR, Galaxy refine, TM-align and SuperPose (for 3D prediction). A part of the *ATM* protein sequence containing exon 37 (NP_000042.3.) was selected and the prediction was carried out based on this region. The effect of nucleotide changes on the splicing at the acceptor site was evaluated by NetGen2 software from CBS prediction services and Human Splice Finder (HSF) system.

Immunofluorescence (IF) Assay

The process of cell extraction, and conjugation was performed according to the previous publications [13, 14]. Three relevant profiles were considered for the IF assay: **Profile A**: Includes: 1. the mouse monoclonal anti-Cyclin E antibody, isotype IgG2b (Invitrogen, USA); goat anti-mouse; 2. Monoclonal mouse anti-ATM antibody, isotype IgG1 (CEDARLANE, Hornby, Ontario, Canada) with goat anti-mouse (GE/Amersham Biosciences); and 3. monoclonal anti-CDC25A (Sigma, USA), with second layer antibody goat anti-mouse (GE/Amersham Biosciences), **Profile B**: Includes: 1. Monoclonal mouse anti-human PCNA, conjugated isotype IgG2a/FITC (DakoCytomation, Denmark); 2. The monoclonal anti-P53 antibody, isotype IgG2b (DakoCytomation, Denmark) and 3. Monoclonal mouse anti-ATM antibody, isotype IgG1. **Profile C**: Includes: 1. Monoclonal mouse anti-human Ki-67, conjugated with FITC, 2. Mouse monoclonal anti-Cyclin D1 antibody, with Rpe, and 3. The monoclonal anti-P53 antibody, isotype IgG2b, with /Pe-cy5. The corresponding mouse immunoglobulin (Dako, Denmark) was used as negative control for the same population of each triple profile. Three blood samples were used as controls which included a sample of the proband and two samples from the healthy subjects in the pedigree who were negative for ATM-D1853N alteration.

RESULTS

Molecular Data

In addition to D1853N polymorphism, the sequencing results of blood and tumor tissues revealed two intronic mutations including IVS 36-46 C>T and IVS 36-8 T>C located within the donor splice site of intron 36 and five coding alterations of V1833M and L1888L, L1842L, H1864H, and S1872R (Figure 1a, b). All the alterations were observed heterozygously in the proband (Figure 1b). D1853N was traced through the pedigree which found to be heterozygously inherited from proband's mother, while the other heterozygote alterations were somatic variations observed in tumor and blood samples of the proband. Furthermore, D1853N polymorphism was also positive in the proband's son who is affected with BC at age of 41.

Cloning Results

D1853N occurred in two different alleles of the proband. Furthermore, both D1853N, IVS 36-8 T>C, V1833M, and L1888L presented in both blood- and tumor- tissue (Figure 1a, b). The other hit- events including IVS 36-46 T>C, L1842L, H1864H, and S1872R were, heterozygously, observed at tumor level, and not in blood sample (Figure 1b). H1864H, S1872R, and L1888L were observed in different allele from allele

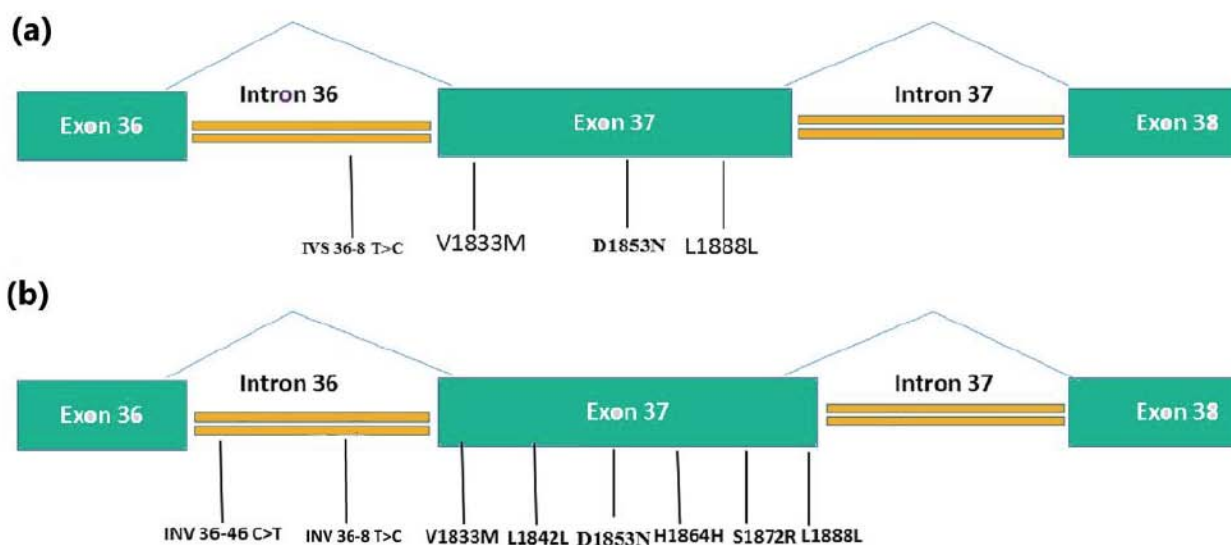


Figure 1: The alterations in Exon 37 and surrounding region of ATM gene.

1a. Sequencing results of blood and tumor tissues present two intronic mutations including IVS 36-46 C>T and IVS 36-8 T>C within the splice site of intron 36 and the coding alterations includes V1833M and L1888L, L1842L, H1864H, and S1872R.

1b. Alterations are heterozygous in the proband.

D1853N, IVS 36-8 T>C, V1833M, and L1888L presented in blood- and tumor- tissue. IVS 36-46 T>C, L1842L, H1864H, and S1872R were, traced in tumor H1864H, S1872R, and L1888L were observed in different allele.

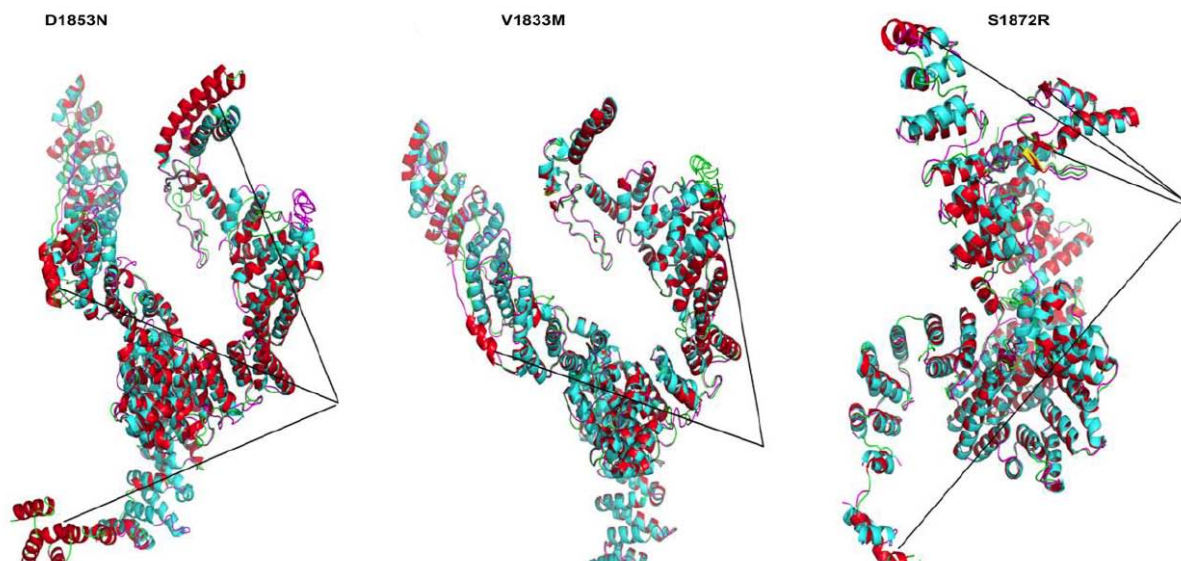


Figure 2: The 3D structure prediction of target region of ATM protein.

The *in silico* analysis showed that each alteration of V1833M, D1853N, and S1872R changes the distribution of helices and loops in the modelled region of ATM. Arrows are indicative of the possible differences introduced by V1833M, D1853N, and S1872R alterations on the 3D-conformation of modelled region.

containing IVS 36-46 T>C, IVS 36-8 T>C, V1833M, L1842L, and D1853N hit events.

***In Silico* Protein Structure Prediction**

The 2D prediction tools showed that coding alterations consisted of D1853N, V1833M, and S1872R. The 2D prediction tools showed that they cause 2D structure alteration in ATM protein through disturbing the percentage and size of sheets, helices and coils. Consistent with 2D, 3D structure alignment of wild-type and mutant-type revealed that the mentioned alterations obviously convey conformational differences on the ATM protein structure (Figure 2). *In silico* analysis by NetGen2 showed that IVS 36-46 T>C could decrease the probability of using the main splice site from 33% to 26%. Also, IVS 36-46 T>C was found to increase the probability of an alternative splice site. HSF matrices predicted that the substitutions of T with C-allele and C with T-allele result in the loss of 3' splice donor site with decreasing the consensus value.

Modification of two-hit hypothesis of tumor suppressor gene and inactivation of E-cadherin [11], and the key role of ATM in development of breast cancer [12] are indicative of the evolving role in molecular cell biology.

Immunofluorescence (IF)

The results of protein expression (PE) assay were provided by three different profiles. Profile A reflects

the PE of Cyclin E, ATM and CDC25A (Figure 3); Profile B includes PCNA, P53 and ATM (Figure 4); and profile C comprises of Ki-67, Cyclin D1 and P53 (S. Figure 4). The follow-up expression assay of cyclin E, ATM and CDC25A in the breast tumor of our proband was, initially, performed the same day after surgery (Figure 3), and followed by the buccal assay after 21 years (S Figure 5).

As profile A reflects, the PE of ATM is low in the majority of cells, but of cyclin E and CDC25A is more diverse (Figure 3a-h). In Profile-B, expression of ATM is low in the majority of cells, and the degree of intensity is diverse in PCNA and P53 (Figure 5a-g). In profile C, Ki-67, cyclin D1, and P53 had a diverse expression (S. Figure 4a-d). The PE is found to be high in cyclin E and CDC25A (Figure 3a-h), and the high expression of cyclin E, ATM, and CDC25A in buccal cells of proband after 21 years of the initial surgery as a primary IDC is remarkable (Figure 4).

DISCUSSION

Carcinoma occurs as a result of the sequential events at genomic and somatic levels including molecular and functional alterations. It is believed that at least 3-7 genetic alterations are required for carcinoma development [9]. There are a few reports where in those the hit evolutionary molecular based pattern in BC has been evaluated, but not in ATM [10-12]. Somatic mutation, loss of heterozygosity (LOH), and methylation have been reported as the hit

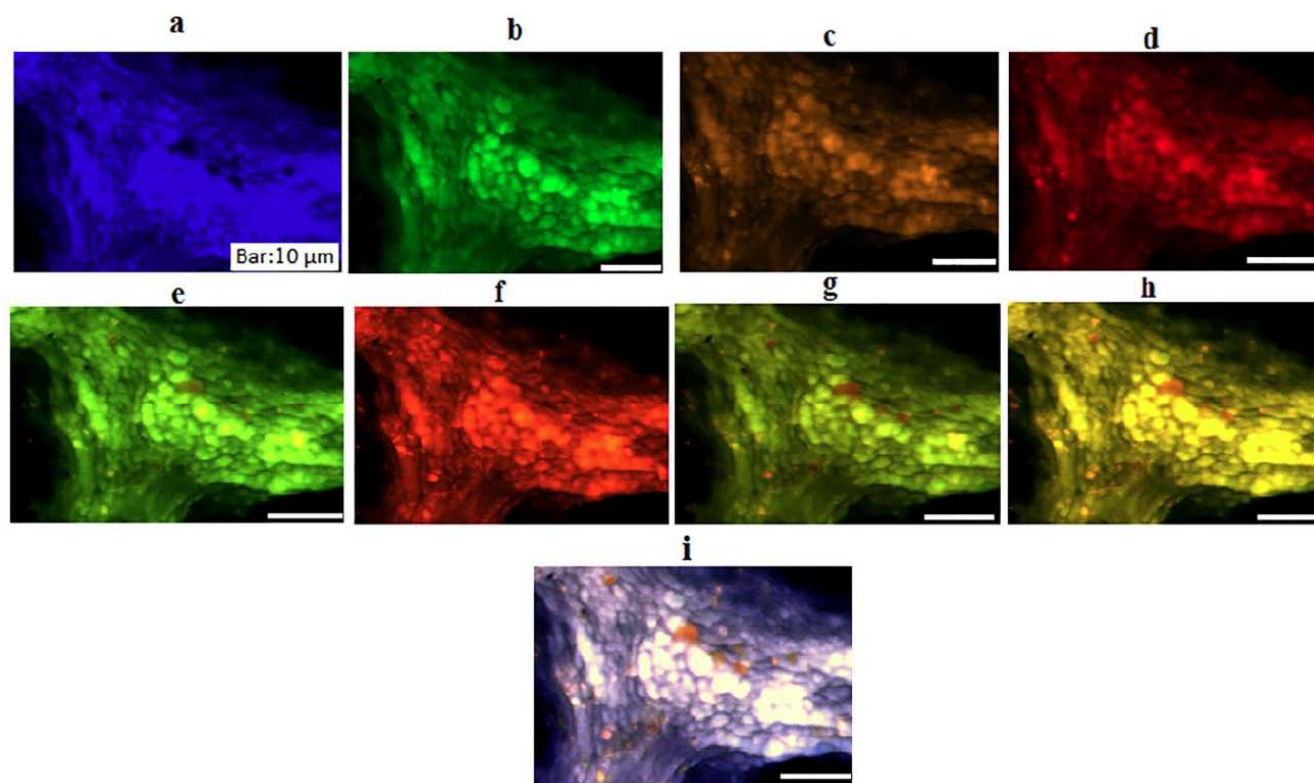


Figure 3: “Protein expression of Cyclin E, ATM and CDC25A in tumor of a patient with breast carcinoma.

a) Breast tumor cells with dapi; **b)** The same cells conjugated with FITC, reflecting the expression of Cyclin E; **c)** The same cells conjugated with Rpe, reflecting the expression of ATM; **d)** The same cells presenting the expression of CDC25A and conjugated with Pe-cy5; **e)** The co-expression of Cyclin E/ATM; **f)** the co-expression of cyclin ATM/ CDC25A; **g)** The co-expression of Cyclin E/CDC25A; **h)** the co-expression of Cyclin E/ATM/CDC25A; **i)** The co-expression of dapi/Cyclin E/ATM/CDC25A. Diverse expression and remarkable co-expression is observable.

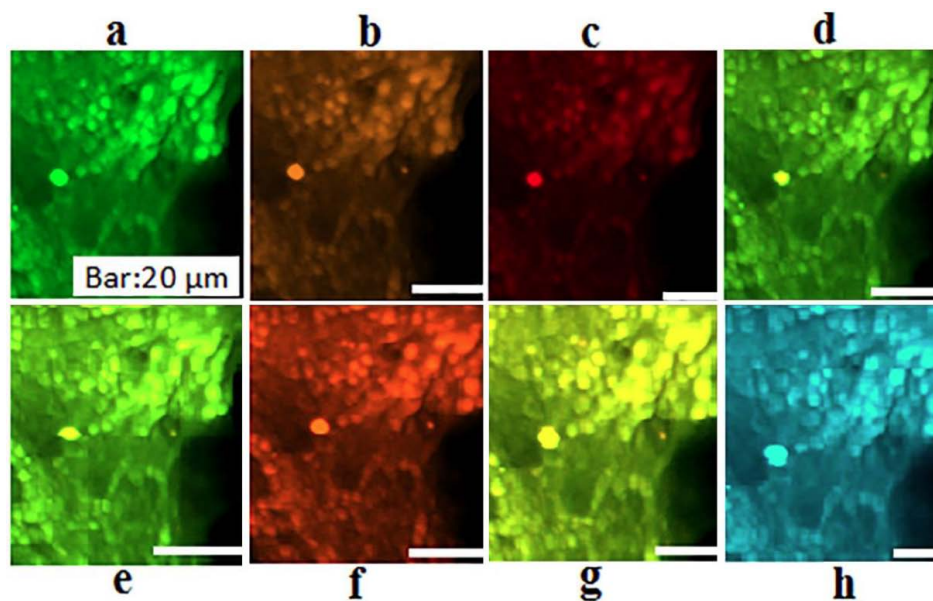


Figure 4: Protein expression of PCNA, P53 and ATM in the tumor of a patient with Breast carcinoma.

a) Breast tumor cells conjugated with FITC, reflecting the expression of PCNA; **b)** The same cells conjugated with Rpe, reflecting the expression of P53; **c)** The same cells conjugated with Pe-cy5, reflecting the expression of ATM; **d)** The same cells presenting the co-expression of PCNA /P53; **e)** The co-expression of PCNA /ATM; **f)** The co-expression of P53/ATM; **g)** The co-expression of PCNA /P53/ATM; **h)** The merged of dapi/P53/ATM

Diverse expression (a-c) and co-expression (d-g) is observable.

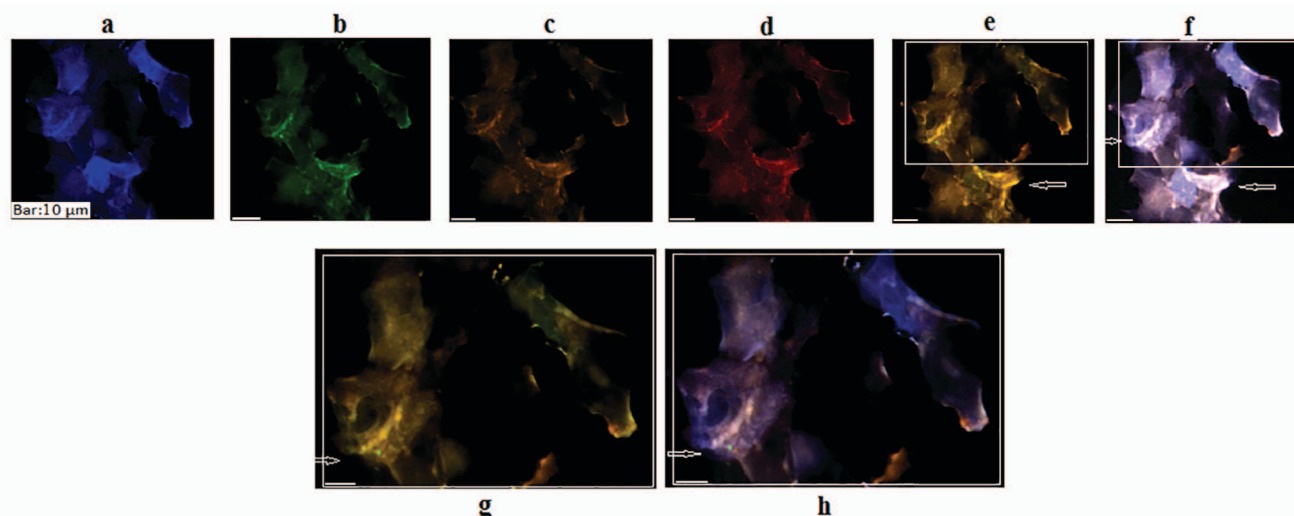


Figure 5: Status of protein expression of Cyclin E, ATM and CDC25A in buccal in a patient affected with breast carcinoma at the same day before surgery

Arrows show the high expression and co-expression of Cyclin E and CDC25A in limited buccal cells 21 years after surgery.

a) Buccal cells with dapi; **b)** The same cells conjugated with FITC, reflecting the expression of Cyclin E; **c)** The same cells conjugated with Rpe, reflecting the expression of ATM; **d)** The same cells presenting expression of CDC25A and conjugated with Pe-cy5; **e)** The co-expression of Cyclin E/ATM/CDC25A in cropped image from; **f)** The co-expression of dapi/Cyclin E/ATM/CDC25A; **g)** The cropped section from image **e**, reflect the co-expression of Cyclin E/ATM/CDC25A; **h)** The cropped section from image **f**, reflects the co-expression of dapi/ Cyclin E/ATM/CDC25A.

contributed to the loss of *ATM* function, however, a real hit is reflective of alteration (s) occurring in a gene and the same related exon [13]. The involvement of *ATM* gene and specifically, the key role of D1853N polymorphism, has been previously reported in different types of carcinomas especially BC and this polymorphism could be screened in a variety of carcinomas [5, 13]. A patent has been also registered by Mehdipour (Application number: PCT/IB2014/065072). The D1853N polymorphism has been highlighted as a predisposing variant [8]. Furthermore, a five-hit hypothesis could be traced in a patient affected with an early onset breast carcinoma [14]. In this patient, five heterozygous 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 exon 37 and its splicing site of *ATM*.

In the current study, we traced D1853N alteration in a proband with BC and evaluated the molecular evolution of hits occurred in exon 37 and its flanking region in *ATM*. The results unmasked the occurrence of the evolutionary alterations through the process of tumorigenesis. At a glance, our findings are reflective of the eight hits including IVS 36-46 C>T, IVS 36-8 T>C, V1832M, L1842L, D1853N (occurred in an allele), H1864H, S1872R (at somatic level) and L1888L (occurred at genomic and somatic level in other allele) in target region of *ATM* of our proband.

In addition to the inherited D1853N polymorphism, the alterations including IVS 36-8 T>C, V1833M, and L1888L were found to be occurred before the differentiation of blood system at early developmental stage, at embryonic period of the proband, i.e., between the beginning of 2nd and the end of 3rd weeks after fertilization of oocyte (S. Figure 6). Interestingly, the tumor-specific presence of IVS 36-8 T>C, V1833M, and L1888L alterations is indicative of the key role of these evolutionary changes through the BC progression either at genomics or tumor level in our patient. This phenomenon has also been previously reported in the three-hit hypothesis in astrocytoma [8]. Surprisingly, further evolution has occurred at tumor level which was found as IVS 36-46 C>T, L1842, H1864H, and S1872R, characterized as the 5th, 6th, 7th and 8th hit events, respectively. The key elements of evolution, is shown in supplementary Figure 2 and Supplementary Figure 3. Apart from the D1853N polymorphism and those alterations which were only observed in breast tumor, the further hit events observed both in blood and tumor tissues of proband could be considered as the early events at a very early stage of differentiation. Therefore, we may conclude that this stage of life is very critical which is, partly, related to the lifestyle of the proband's mother and other factors including the predisposing capability of the proband.

In overview, either environmental factors during the proband's embryonic life, or genetic susceptibility of a triangle partner including parents or embryo could be highlighted. In this case, the influential advice is essential to be provided to the couples. These are the matter of an early systematic and applicable strategy which seems to be the key elements for carcinoma management. In the previous report conducted by Mehdi-pour and her colleagues, the heterozygous D1853N polymorphism was proposed as an essential molecular alteration for successful evolutionary events in the astrocytoma. Furthermore, two additional intronic hit events at the splicing site of *ATM* gene at Exon 37 were aimed to progress the tumorigenic event by completing the evolutionary process [5]. The probability of pathogenic effects of reported hit events was analyzed *in silico* based on some bioinformatics tools such as SIFT, PolyPhen, MUpro, and Project HOPE. According to the COSMIC catalogue and bioinformatics tools, D1853N is considered as a predisposing coding variant that is located at a crucial region and decreases the stability of protein structure. The mutant amino acid has a different charge that can cause loss of interactions with other molecules or residues. However, this genetic alteration is predicted to be benign based on the dbSNP (<https://www.ncbi.nlm.nih.gov/snp/rs1801516>), but with a predisposing capability. Our clinical and molecular findings also confirmed the pathogenic effects, therefore, D1853N is considered as an initiator hit to support tumorigenesis of deficient *ATM* in proband which is confirming the previous reports [15-17]. There were two other missense alterations including V1833M and S1872R. According to *in silico* analysis, these alterations are predicted to be "POSSIBLY DAMAGING" due to the differences in size, charge, and hydrophobicity between wild-type and mutant amino acids. Supporting the mentioned data, comparison of 2D (data are not presented) and 3D structure model of the selected region containing D1853N, V1833M and S1872R showed an obvious change between the wild-type allele and the mutant one, that consequently might introduce function deficiency and pathological effects (Figure 1b).

Since the number of each tRNAs related to each amino acid codon is not equal in the tRNA pool of the cell, therefore, a synonymous change could also have an adverse impact on the speed and level of translation and consequently the level of protein production. This phenomenon is reflected "as codon usage bias" [18, 19]. Accordingly, substitution of CTT with CTC (L1842L), substitution of CAT with CAC (H1864H), and substitution of CTT with CTC (L1888L) may have an

inverse impact on *ATM* protein production and finally exert the pathological effect. Interestingly, the *ATM* gene harbor the predisposition capability to be evolved with more aggressive behavior than an oncogene.

In addition to the invariant 5'-GT and 3'-AG splicing sites, the extensive consensus sequence spanning 5' and 3' splice sites is also a determining factor of an accurate splicing [20, 21]. Consequently, variations affecting splicing are expected to compromise a noticeable proportion of genetic disease etiology [22]. IVS 36-8 T>C (rs3092829) and IVS 36-46 C>T were the splicing region variants. Although the most straightforward and reliable method to detect splicing defects is the analysis of RNA, *in silico* analysis by NetGen2 and HSF matrices disclosed the adverse effect of these alterations on splice efficacy and consequently, these intronic hit events can have pathological impacts as well.

Regarding the *in silico* analysis, predicted score should be interpreted with caution and further *in vitro* studies are required which may address the possible impact of coding and splicing variants on function and expression of *ATM* protein [23]. The mitotic cell cycle is progressed through a reproducible sequence of events that are regulated by different kinds of proteins. When Eukaryotic cells are exposed to DNA damage, a signaling pathway will be directed by *ATM* through which the activation or inactivation of the involved genes including *P53* in either G1 or G2 would occur. Besides, degradation of CDC25A protein phosphatase, as an intra-S-phase checkpoint consequence, is considered as a key event during the cell cycle [24].

Focusing on the functional insight, it was previously reported that the PE of CDC25A and cyclin E were significantly correlated with Ki-67 expression level [25]. Furthermore, no clear data on the co-expression and impact of these three genes on the clinical outcome of BC are available. However, it is expected that the status of PE in CDC25A, Cyclin D1, and cyclin E would remain high, but PE in *P53* will be constantly low in carcinoma cells. In addition, due to the FH of the brain tumor in proband's second cousin, the issue of Li-Fraumeni's syndrome was considered, however, no mutation was detected in *P53*.

In our patient, regarding the profile-A, the low PE of *ATM* in the majority of cells confirms the diverse molecular alterations as eight-hit. However, cyclin E and CDC25A revealed to have more diverse pattern of PE (Figure 3a-i).

Furthermore, the co-expression between proteins of ATM/CDC25A was remarkable for few cells, but more harmonic cooperation is detectable between cyclin E and CDC25A (Figure 3d) which is facilitating more aggressive behavior of the BC tumor. These functional data are due to the diverse molecular alteration and the priority of essential and required genes for the survival of BC cells. This explanation is also indicative of a severe heterogeneity at the cellular level.

In Profile-B, degree of intensity in majority of cells is revealed to be high for PCNA and moderate in P53 which is indicative of the high proliferation and the relative influential role of P53 as the cell guardian respectively. But, the PE of ATM is low in the majority of cells with a harmonic co-expression with P53 (Figure 3a-d). Besides, there is a prominent single cell with harmonic triangle cooperation due to the high expression of ATM and P53 which may have an influential positive impact on the tumor status by restricting and/or balancing the progression of BC tumor by PCNA (Figure 4b-d). In spite of the occurrence of an evolutionary eight-hit events and based on the current 21 years' survival period of the proband, the presence of a minor clone, with a normal function of ATM and higher expression of P53, had a remarkable positive impact on the health status and a satisfactory quality of her life.

In Profile-C, Ki-67, cyclin D1, and P53 have a diverse expression, however Ki-67 and cyclin D1 reflect a harmonic cooperation (S. Figure 4). Although the expression of P53 is low in this profile, some cell population with high PE may indicate the resistance of P53 to rescue the target cells from more invasive behavior (S. Figure 4d).

Furthermore, the initial assay of PE in the buccal cells of the proband indicated the high expression of cyclin E and CDC25A in few cells with a negative impact on the course of disease in our BC patient (Figure 5a-h). The follow-up study on her buccal cells reflects the diverse and low expression of ATM, cyclin E, and CDC25A after 21 years of the initial surgery of the proband but with noticeable co-expression (S. Figure 5a-f).

In conclusion, our findings reflect the sequential molecular and cellular events through the entire patient's life from the pre-differentiation embryonic stage and all through the post-birth periods. In spite of such events, our patient is also living in a stable psychological status. Finally, these data are reflective of a personalized diagnosis that would lead to manage the early preventive strategies, either for the patient or

for the relatives of proband. Moreover, as such hit events may occur in other carcinoma patients, similar studies may be applied as a screening test for an appropriate personalized carcinoma management and may lead to an innovative therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS CERTIFICATE

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Project number: 95-04-30-32208

Principal investigator: Professor Parvin Mehdipour

Approval date: 08/03/2017

CONSENT TO PARTICIPATE

A signed informed consent form (according to the Declaration of Helsinki) was taken from proband.

AUTHORS' CONTRIBUTION

Parvin Mehdipour contributed to the design of study; analyses of data; configuring the molecular sequential cascade, and manuscript edition. Asaad Azarnezhad was involved in laboratory works, data analysis, bioinformatics analysis, and manuscript writing.

COMPETING INTERESTS

The authors disclose any financial or non-financial interests that are directly or indirectly related to the present work submitted for possible publication.

SUPPLEMENTARY FIGURE

The supplementary Figures can be downloaded from the journal website along with the article.

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