Original Article

Variation of *ATM* Gene Expression in Peripheral Blood Cells of Sporadic Breast Carcinomas in Iranian Patients

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Abstract

The ataxia telangiectasia mutated gene (*ATM*), candidate for breast cancer susceptibility gene, encode a 350-*kDa* protein belongs to the core components of DNA-damage response machinery. Female *AT* carriers have at least 5-fold increase risk for breast cancer. Reduction in *ATM* expression is shown in multiple studies in breast tissues. We aimed to perform a research to measure the *ATM* mRNA expression in peripheral blood cells in breast cancer patients. Peripheral blood sample from 40 newly diagnosed, histologically confirmed female breast cancer patients was collected before surgery. Total RNA was isolated from blood cells using the RNX-Plus solution and reverse transcribed into cDNA. Real-time PCR was performed using the $2^{-\Delta\Delta CT}$ method to calculate relative changes in gene expression by REST software. The Relative Quantitation (RQ) mean was 1.27 with the min. and max. equal to 0.20 and 3.34, respectively. Calculation of patient frequencies in different groups revealed that 17.5% had reduced expression lower than two fold decreases and 15% high expression more than two fold increases, but according to REST software there was no up-regulation or down-regulation compared to normal females. The findings of multiple studies consistent with this study indicate that the *ATM* gene may play an important role in breast cancer development and progression, and *ATM* expression is down-regulated in breast cancer tissues. Although, some of the results do not support a suppressor role for *ATM* in the development of sporadic breast cancer, 17.5% of our patients had under expression of *ATM* mRNA less than two fold relative to control.

Avicenna J Med Biotech 2012; 4(2): 95-101

Keywords: Blood cells, Breast neoplasms, mRNA, Polymerase chain reaction, Real-time

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Received: 7 Dec 2011
Accepted: 11 Mar 2012

Introduction

Several genes are known to predispose women to breast cancer, which is a common disease with a complex etiology. Although mutations in *BRCA1* and *BRCA2* are recognized as risk factors for inherited breast cancer, somatic mutations in these genes are rare in sporadic breast cancers [1,2]. In principle, a greater proportion of breast cancer cases with in the population could be attributed to genes that are more frequently mutated but which may have a relatively low penetrance with respect to breast cancer [3,4].

The Ataxia Telangiectasia Mutated (*ATM*) gene is one candidate for such a susceptibility gene. The *ATM* a 350-*kDa* protein serine/threonine kinase, member of the phosphoinositide 3-kinase (PI3-kinase)-like family (PIKK) belongs to the core components of DNA-damage response machinery and acts as an intracellular sensor by recognizing double-

strand breaks (DSBs). Numerous substrates involved in DNA repair are regulated by *ATM* protein kinase activity. *ATM* is the initiator of a signaling cascade that responds to DSB and thought to be master controllers of cell-cycle checkpoint signaling pathways [3,5,6].

Mutation of the *ATM* gene is the underlying cause of the rare autosomal recessive disorder Ataxia Telangiectasia (*AT*), characterized by clinical manifestations that include progressive cerebellar ataxia, skin, and ocular telangiectasia, immunological deficiency, neuronal degeneration, extreme cellular sensitivity to ionizing radiation (IR), premature ageing, hypogonadism, growth retardation, and predisposition to cancer. *AT*, described as a separate disease in 1957, occurs early in childhood with a frequency varying from 1 in 40,000 to 300,000 births in various ethnic groups [7,8].

The *ATM* gene was mapped to chromosome 11q22–23 in 1988 [9] and a single *AT*-mutated gene was identified in 1995 [2]. More than 400 disease-causing mutations have been identified in *ATM* so far and about 70% of them result in premature termination of translation and truncation of the protein. 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* [5].

An estimated 1.4% of the population carries a single ataxia-telangiectasia gene. Epidemiological studies on *AT* families have shown that *AT* heterozygotes also have an increased risk of developing cancer, in particular breast cancer, for which female *ATM* carriers have at least 5-fold increase risk compared with the general population. Seven percent or more of all breast cancer patients are likely to carry a single ataxia-telangiectasia gene. This gene appears to predispose carriers to breast cancer primarily with onset before age 65 or 70 years. Thus, the proportion of ataxia-telangiectasia heterozygotes among women with early breast cancer onset may be substantially greater than 7% [10,11], but its true magnitude is still uncertain [12].

Normal breast tissue shows a distinct pattern of *ATM* expression, the protein being found in the ductal epithelial cells but not in the surrounding myoepithelial cells. In contrast, in cases of sclerosing adenosis, a benign breast lesion, *ATM* is expressed in both the epithelial and myoepithelial cells. This up-regulation of *ATM* expression was associated with proliferation of the myoepithelial cells [13].

Several groups studied large cohorts of sporadic breast cancer patients and age-matched controls for nonsense or frame-shift mutations within the *ATM* gene. Neither group found evidence for a higher incidence of defective *ATM* alleles in the cancer patient cohort. Furthermore, the correlation between breast cancer and dominant negative forms of *ATM* arising from defined missense or intronic mutations within the *ATM* gene remained controversial. It seems that inactivation of the *ATM* gene by somatic mutations is not a common hallmark of breast cancer [5,14].

A role for the *ATM* gene in sporadic breast cancer is supported by many studies that have shown a LOH in the region of the *ATM* gene. This has been found in 40% of tumors studied. A causative association with the *ATM* gene has, however, been shown in only a few familial cases of breast cancer [3,15-17]. The epigenetic silencing of *ATM* expression occurred in locally advanced breast tumors suggesting a link between reduced *ATM* function and sporadic breast cancer [14].

HER2, Estrogen (ER) and Progesterone (PR) Receptors are currently used in routine patient care of breast cancer [18]. Over expression of human epidermal growth factor receptor type 2 (HER2), a 185-*kD* receptor occurs in 20 to 30% of invasive breast cancers. In general, tumors with a HER2 gene amplification have decreased overall survival and may respond to targeted therapy [19]. ER positive tumors considered as lower penetrance alleles are thought to have characteristics of the luminal cell type and are frequently responsive to endocrine treatment. The PR is a prognostic marker, but the Oxford overview

of adjuvant therapy does not support its ability to predict resistance to chemotherapy [20,21].

Multiple studies evaluated *ATM* gene expression in breast tissues, but so far, there was no available study on mRNA expression in tissues other than breast to determine a probable constitutional genetic predisposition factor in these patients. However, the expression of *ATM* gene in peripheral blood lymphocytes was evaluated in some cancers including bladder and lung [22,23]. We aimed to perform a research to measure the *ATM* mRNA expression in peripheral blood cells in breast cancer patients and also their correlations in different groups.

Table 1. Characteristics of patients with breast cancer

Character	No. of subjects	Percentage
TNM stage		
T0	1	2.5
T1	18	45
T2	14	35
T3	3	7.5
T4	4	10
N1	23	57.5
N2	13	32.5
N3	4	10
ER/PR status		
ER-positive	30	75
PR-positive	24	60
HER2 Positive	9	22.5
Total	40	100
Mean of age (±SD)	48±11.5 *yr*	

Materials and Methods

Study population

This study was approved by the local ethical committees. For the study, 40 newly diagnosed, histologically confirmed female breast cancer patients from Imam Khomeini hospital (Tehran, Iran) undergoing surgery were recruited between 2010 and 2011. There were no age, ethnicity, and tumor pathology or stage restrictions. Thirty three (83%) samples were invasive ductal carcinoma, 16 (48%) of these tumors were associated with an *in situ* ductal carcinoma. One sample was exclusively an *in situ* ductal carcinoma and 6 (15%) samples were other malignant breast tumors. None of the patients was treated with radiotherapy or chemotherapy before surgery. Twenty four control subjects with no prior history of cancer with the same gender for patients were recruited. The general characteristics of cancer patients are listed in table 1.

Blood sample collection

Peripheral blood sample from each participant was collected into EDTA tube before surgery and then transported immediately to the laboratory where the specimens were processed.

RNA extraction and cDNA synthesis

Total RNA was isolated from blood cells using the RNX-Plus solution for total RNA isolation (RNX-Plus solution; Cinnagen, CN. RN7713C) according to the manufacturer's protocol. The kit utilizes the phenol chloroform method. Total RNA was eluted with 15 μl DEPC-treated water. One μl RNase inhibitor was added to prevent degradation of RNA (RiboLock^{TM} RNase inhibitor; Fermentas Life Sciences). RNA concentration and purity were determined on a UV spectrophotometer by the 260 *nm* absorbance and 260/280 *nm* absorbance ratio, respectively. One μg of total RNA per sample was reverse transcribed into cDNA using the cDNA synthesis kit (QuantiTect Reverse Transcriptase kit; Qiagen, CN. 205311) according to the manufacturer's protocol.

Quantitative real time PCR

The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. The probes and primers used for real-time RT–PCR were designed using the Primer Express software (version 2.0; Applied Biosystems) and synthesized by Takapou Zist Co. (Tehran, Iran). To avoid amplification of the contaminating residual genomic DNA, the probe and primer sets for each gene were designed around the junction region of two exons so that they were mRNA-specific. Human β actin gene was used as internal control to normalize input RNA amount, RNA

Table 2. Primer and probe sequences used in real-time PCR

Gene	Forward (F) and reverse (R) primers, probes (P)(5'- 3')	Reporter/Quencher	Length (mer)
ATM	F: CTC TGA GTG GCA GCT GGA AGA R: TTT AGG CTG GGA TTG TTC GCT P: CAC AGC TGG CAT CCA ACT TCT TGA TCA TT	FAM/TAMRA	21 21 29
β actin	F: CAG CAG ATG TGG ATC AGC AAG R: GCA TTT GCG GTG GAC GAT P: AGG AGT ATG ACG AGT CCG GCC CC	FAM/TAMRA	21 18 23

quality and reverse transcription efficiency. The sequences of primers and probes are listed in table 2.

Real-time PCR was performed using the Applied Biosystems 7500 real-time PCR system according to the manufacturer's protocol. Typical amplification mixtures (20 μl) contained the sample DNA (cDNA); 10 μl Taq Man® Universal PCR Master Mix (Roche Molecular Systems, Inc.; Part No. 4318157, New Jersey USA); forward and reverse primers and probes.

The efficiency of primers was 101.6 and 100.2 for *ATM*, and β actin, respectively. The thermal cycling conditions consisted of 1 cycle for 10 *min* at 95°*C* to activate the Ampli Taq Gold enzyme, and 45 cycles for 15 *sec* at 95°*C* and for 30 *sec* at 60°*C*. The quantitative PCRs were performed in duplicate for each sample, and the mean was used as the RQ value. The fold change in the *ATM* gene expression in patients ($2^{-\Delta\Delta CT}$=RQ) normalized to β actin and relative to the expression at control samples was calculated for each sample where $\Delta\Delta CT = (C_{T,ATM} - C_{T,\ \beta\ actin})_{Patient} - (C_{T,ATM} - C_{T,\ \beta\ actin})_{Control}$ [24].

Statistical analysis

The Independent-Samples t-test or one-way ANOVA test was used, when appropriate to compare quantitative data. For evaluating relative expression of *ATM* mRNA in patients, REST (relative expression software tool) 2009 V2.0.13 was used. The statistical analysis was performed with the program SPSS 16.0 for windows.

Results

Real-time RT-PCR of *ATM* mRNA expression in peripheral blood cells of patients showed the RQ mean equal to 1.27 (SD=0.76). The min. and max. was 0.20 and 3.34, respectively. Calculation of patient frequencies in different groups of RQ (Figure 1) revealed that 17.5% had under expression less than two fold decrease and 15% over expression more than two fold increase, but according to REST software there was no up-regulation or down-regulation compare to normal females. No correlation was found between *ATM* expression and tumor size (p=0.563), lymph node involvement (p=0.847), ER or PR (p=0.235 and p=0.605 respectively), or HER2 status (p=0.295). There was no significant correlation between RQ and different groups of ages, as well. Figure 2 shows amplification plot of some tumor and control samples.

Discussion

The *ATM* protein kinase, encoded by the *ATM* tumor suppressor gene, is the apex of the repair pathway of DSB, a serious lesion occurring in DNA [5]. *ATM* protects the integrity of the genome at different levels: (1) it mediates arrest of the cell cycle at G1/S, S,

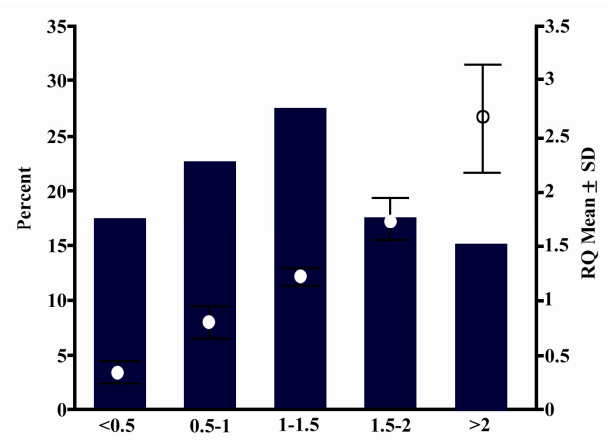

Figure 1. RQ mean and frequencies of patients in different RQ groups.

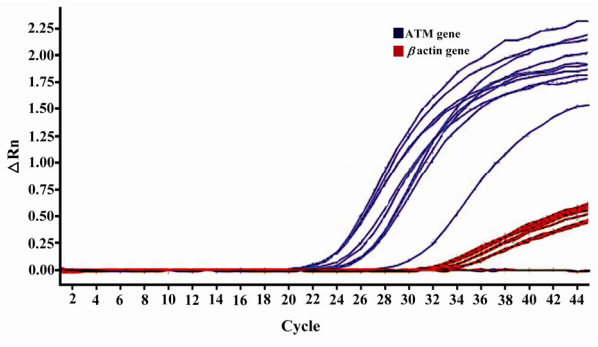

Figure 2. Amplification plot of some tumor and control samples

and G2/M to prevent the processing of damaged DNA; (2) it activates DNA-repair pathways; and (3) it induces apoptosis if the DNA damage is so detrimental that normal cell function can no longer be rescued [7].

The findings of multiple studies are consistent with the hypothesis that the *ATM* gene may play an important role in breast cancer development and progression, and also *ATM* expression is down-regulated in breast cancer tissues [20]. Multiple studies revealed that the *ATM* mRNA level was significantly lower in breast cancer tissue than the adjacent normal tissue from the same patients or breast tissues from patients with benign breast diseases [25,26]. This reduction was observed in breast tumors with or without LOH in the region of the *ATM* gene, suggesting that genetic events other than gene deletions could result in reduced ATM gene expression [27]. However some studies showed that low expression of *ATM* in breast cancer tissue was related to a high rate of DNA mutation in cancer cells and a progressive cancer phenotype [28].

In some other studies *ATM* expression levels were the lowest in breast cancer tissue, compared to benign tumor tissue and normal breast tissue specimens [3,6,14]. Reduced *ATM* expression was also associated with increased neovascularization in breast cancers, but a cause-effect relationship is yet to be demonstrated [6]. Further, Vo et al found a highly significant correlation (p=0.0006) between reduced *ATM* mRNA abundance, as measured by real-time RT–PCR, and aberrant methylation of the *ATM* gene promoter. These findings indicate that epigenetic silencing of *ATM* expression occurs in locally advanced breast tumors, and establish a link at the molecular level between reduced *ATM* function and sporadic breast malignancy [14]. But Luo et al have shown that in lymphocytes expressing *ATM*, the promoter region is completely demethylated. However, they were unable to correlate the methylation status and the variable *ATM* protein expression [5,29].

These results indicate that *ATM* down-regulation might be important at different levels and by different mechanisms in mammary carcinogenesis and that it may significantly contribute to the pathogenesis of breast carcinomas. Although, the results of Kovalev et al experiment do not support a suppressor role for *ATM* in the development of sporadic breast cancer [30].

ATM has more expression in higher grades of astrocytoma tumors [28], but in breast cancer patients high level of *ATM* expression in breast cancer tissue was linked to improved survival [25]. *ATM* expression is a complex process, which is influenced by several reasons including DNA damage, accelerated protein degradation, mRNA instability, hyperphosphorylation of effectors proteins in the protein synthesis machinery [23].

Our results show no up- or down-regulation of *ATM* mRNA in peripheral blood cells in breast cancer patients, but in some other cancers reduced expression was seen. Jianlin et al evaluated the *ATM* protein expression levels of lung cancer patients in peripheral blood lymphocytes, and displayed that the *ATM* protein expression levels of lung cancer patients were significantly lower than that of controls [23]. Among bladder cancer subjects the expressions of *ATM* was significantly lower in cases than in controls (p=0.04). A few studies showed that mRNA expression level of DNA repair or methylation-related gene in peripheral blood lymphocytes is associated with altered cancer risk. For example, reduced expression of mismatch repair genes and nucleotide excision repair genes confers significantly increased risk for head and neck cancer

[22]. However, our data correlate with Ye et al (2007) that no significant association was observed between *ATM* expression level and TNM stage [25].

ATM under expression was more observed in triple-negative breast tumors ER(-), PR(-) and HER2(-) [31]. Furthermore, in another study the non-BRCA1/2 tumors with reduced *ATM* expression were more often ER negative, and PR negative [32], whereas according to our findings *ATM* expression in peripheral blood cells show no correlation in ER/PR status.

As shown in figure 1, 17.5% of patients had under expression of *ATM* mRNA less than two fold relative to control. The importance of this reduction should be evaluated in complementary studies that compare *ATM* expression in breast tissue and peripheral blood cells simultaneously.

Acknowledgement

This study was financially supported by the Research Department of the Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. The authors would like to thank Mrs. Esmaeili for her technical help.

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