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ORIGINAL ARTICLE

Simultaneous *ATM/BRCA1/RAD51* expression variations associated with prognostic factors in Iranian sporadic breast cancer patients

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Abstract
Background DNA double-strand breaks (DSBs) as a serious lesion are repaired by non-homologous end-joining and homologous recombination pathways. *ATM*, *BRCA1*, *RAD51* genes are involved in HR pathways. While some studies have revealed individual expression changes of these genes in different types of cancer, there are limited studies attempting to evaluate correlation of expression variations of these genes in breast cancer pathogenesis. This study aimed to determine *RAD51, ATM* and *BRCA1* gene expression level and its association with clinicopathological factors in fresh breast cancer tissues. Moreover, this study evaluates potential correlations among expression levels of these genes.
Methods 50 breast cancer tissues were collected and examined for *BRCA1, RAD51* and *ATM* gene expression by Real Time PCR. Expression changes were analyzed with REST software version 2009.
Results mRNA expression was reduced in all these three genes when compared with *β-Actin* as a control gene ($P_{value} < 0.001$). Spearman's test demonstrated a significant positive correlation among *ATM*, *BRCA*1 and *RAD*51 gene down expression ($P_{value} < 0.0001$). There was a significant association between down expression of *ATM* with stage ($P_{value} < 0.05$), necrosis ($P_{value} < 0.05$), perineural invasion ($P_{value} < 0.05$), vascular invasion ($P_{value} < 0.01$), malignancy ($P_{value} \leq 0.001$), *PR* ($P_{value} < 0.05$) and *ER* status ($P_{value} < 0.01$). In addition, there was a significant association between down expression of *BRCA1* with *Ki67* ($P_{value} \leq 0.001$). Moreover, there was a significant association between down expression of *RAD51* with lymph node involvement ($P_{value} < 0.01$), auxiliary lymph node metastasis ($P_{value} = 0.01$), age ($P = 0.001$), grade ($P_{value} < 0.05$) and *PR* status ($P_{value} < 0.05$).
Conclusion This study suggests association between expression changes in several DSB repair genes in a common functional pathway in breast cancer and the significant association between abnormal expression of these genes and important clinical prognostic factors.

Keywords ATM · BRCA1 · RAD51 · Breast cancer · Real Time PCR

Introduction

Breast cancer is the major cause of cancer-related mortality among women [1]. Double-Stand Breaks (DSB) are the most severe type of damage. Homologues Recombination pathway (HR) is an extensively regulated process that is involved in the repair of DSB. Deficiencies in DSB repair increase the risk of breast cancer [2]. ATM (Ataxia telangiectasia mutated), BRCA1 (Breast cancer 1), RAD51 are three key proteins involved in the control of HR pathway. ATM is activated in response to DSB and triggers cellular signaling pathways [3]. Once activated, ATM in turn activates its target genes by phosphorylation. *BRCA1* is one of these target genes [4].

BRCA1 takes part in HR and Non Homologues End Joining (NHEJ) repair. *BRCA1* deficient cells are inefficient in repairing DNA damage by HR [5] and the most

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used pathway in these cells is NHEJ, which is an error-prone repair pathway [6]. Association of BRCA1 with RAD51 provides the first evidence that *BRCA1* participated in DNA repair [7].

RAD51 is a central recombinase in HR that in preparation for HR, forms nucleofilament on ssDNA[1] which then promotes strand invasion and homologous pairing between two DNA duplexes [8]. It has been reported there is a reduced expression of *BRCA1* and *ATM* in breast cancer [6, 8, 27]. However, albeit the important role of ATM as a sensor of DSB and initiator HR repair, there is no investigative report examining the correlation of expression changes of *ATM* with *BRCA1* and *RAD51* expression.

Human BRCA1protein shows general cytoplasmic and nuclear expression. RAD51 has high nuclear expression in proliferating cells and low nuclear and cytoplasmic expression in most other cells. ATM protein is predicted to have nuclear localization [9].

In this study our purpose was to investigate the expression of these genes in breast cancer using Real Time PCR and examine whether there was a correlation among the expression changes of *ATM*/*BRCA1*/*RAD51* in sporadic breast cancer. In addition, we aimed to assess the association of *ATM, BRCA1*, and *RAD51* gene expression with prognostic factors.

Materials and methods

Tissue collection

Breast tumor and adjacent normal tissues were removed surgically from 50 women patients admitted to the Khatamolanbia Hospital and exposed to a mastectomy from 2011 to 2015. Fresh tumor and normal adjacent ones at the margin of the tumors (2–3 cm distance) containing normal mammary gland tissues were collected by the clinicians in separate sterile tubes. Tissue samples were frozen and stored at −70 °C. Two pathologists confirmed the cancerous tumors and normal tissues. Staging of the breast cancer was performed according to the Union for International Cancer Control (UICC) which is based on (AJCC-TNM) classification.

Primers and PCR consumables

The cDNA sequences of *ATM, BRCA1, RAD51* and *β-Actin* genes were obtained from Gene Bank. After identifying the exon/intron junctions, upper primer was selected in exon/intron boundaries. The selected sequences were evaluated by OLIGO 6 for hairpin and duplex formation stability. Lower primers were selected using Primer3 program (http://frodo.wi.mit.edu/primer3/primer3_code.html). BLASTN searches against dbEST and nr were conducted to approve gene specificity of the primer sequences and the absence of DNA polymorphisms (Table 1).

RNA Extraction and cDNA synthesis

TriPure Isolation Reagent (Roche Applied Sciences) was used for total RNA extraction of breast tissue samples. Electrophoresis, through agarose gels and ethidium bromide staining, was used to determine the quality of the RNA samples. The concentration of RNA was measured by Nano Drop spectrophotometer. After synchronizing all samples, 1 μg of RNA from each sample was used to synthesize cDNA using First Strand cDNA Synthesis Kit, Fermentas, USA.

Real time PCR

The Relative quantification of *ATM, BRCA1, RAD51* and *β-Actin* transcripts were carried out in samples using Light Cycler TM system (Rotor gene, Corbett, Germany) and Fast-Start DNA Master SYBR-Green I kit (Roche Germany) with specific primer. *β-Actin* was used as an internal control. The PCR was performed in 10 μL of reaction mix, containing 2 μL of Master Solution, 0.3 μM of each primer and 2 μL of cDNA as a template which was placed into 0.1 vials. Thermal cycling consisted of an initial denaturation step, 95 °C for 5 min, followed by an amplification program. Cycling conditions were 95 °C for 5 min, 45cycles with 3 step (1) 95 °C for 10 s (2) 62 °C for 15 s for *ATM* and *RAD51* and 61 °C for 15 s for *BRCA1* (3) 72 °C for 15 s. To evaluate the efficiency of each reaction precisely, we use Linreg software.

Statistic analysis

Real Time PCR data analysis was performed by REST 2009 software. All data are demonstrated as the mean ± standard error (SE) of triplicate experiments. The association between the changes in gene expression and main clinicopathological items was assessed by SPSS software (version 22.0) with Student's *t* test and ANOVA. In the current study, P_{value} less than 0.05 ($P < 0.05$) was considered statistically significant.

Result

Patients Characteristics

Pathology examinations showed that 12 (24%), 23 (46%) and 12 (24%) patients were related to grade of I, II, III,

[1] Single-Strand -DNA.

Table 1 Primers used for RT-PCR amplification *ATM*, *BRCA1*, *RAD51* and *β-actin*

	Primer sequence	Amplicon size (bp)	Accession number
ATM-F	5′-CAGAAGACAGCGATCCAGTG-3′	198	NM_000051.3
ATM-R	5′-GTGCCAGAATGTGAACACCA-3′		
BRCA1-F	5′-GGATTTTCGGGTTCACTCTG-3	229	NM_007294.3
BRCA1-R	5′-CCAAAAGGAGCCTACAAGAAAG-3′		
RAD51-F	5′-CGCTGATGAGTTTGGTGTAGC-3′	245	NM_133487.3
RAD51-R	5′-CATCTCCCACTCCATCTGCA-3′		
β-ACTIN-F	5′-GAGACCTTCAACACCCCAGCC-3′	161	NM_001101.3
β-ACTIN-R	5′-AGACGCAGGATGGCATGGG-3′		

respectively. Thirty-two (62%) of the patients expressed *ER* and 29 (58%) of them expressed *PR*. In addition, 19 (38%) of the patients were *HER2* positive (Table 2). According to pathological reports, patients were *P53* and *BRCA1* mutation negative. No one was exposed to neoadjuvant chemotherapy before surgery.

PCR optimization and validation

Melting curve analysis showed only one peak for each reaction. Gel electrophoresis also showed a single band product with the desired length (Supplementary data, Fig S1).

ATM gene expression analysis in normal and tumor tissues

ATM gene expression analysis in normal and tumor tissues

Analysis of Real Time PCR results by REST 2009 revealed a significant reduction in gene expression level of *ATM* by 7.1 fold in tumor tissues (0.1709 ± 0.0276) in comparison with corresponding adjacent normal samples (1.2240 ± 0.1386) (Fig. 1). One way ANOVAs revealed a significant association between down expression of *ATM* and the stage of tumor. ($P_{value} < 0.05$). Patients in stage I demonstrated the lowest mean expression (0.1045 ± 0.0209) in comparison with stage II (0.1982 ± 0.0210) and stage III (0.2081 ± 0.0366) (Fig. 2a; Table 2). In addition, there was a significant association between down expression of *ATM* and ER status ($P_{value} \leq 0.01$). Mean expression in ER negative group showed significant decrease (0.1046 ± 0.0130) when compared with ER positive group (0.2496 ± 0.0347). Furthermore, there was a significant association between decreased expression level of *ATM* and PR status ($P_{value} < 0.05$). Mean expression in PR negative group (PR−) was lower (0.1078 ± 0.0229) when compared with PR positive (PR+) group (0.2507 ± 0.0237) (Fig. 2b; Table 2). There was also a positive significant association between down expression of *ATM* and necrosis ($P_{value} < 0.05$). Mean expression in groups with tumor necrosis was 0.0515 ± 0.0040 and in group without necrosis was 0.2794 ± 0.0184 (Fig. 2c; Table 2). *T* test analysis showed the significant association between down expression of *ATM* gene with perineural invasion ($P_{value} < 0.05$) (Fig. 2d) vascular invasion ($P_{value} < 0.01$) (Fig. 2e), and malignancy ($P_{value} \leq 0.001$) (Fig. 2f; Table 2).

BRCA1gene expression analysis in normal and tumor tissues

A remarkable decrease by 20 fold was observed in the expression level of *BRCA1* in tumor tissues (0.1979 ± 0.0458) when compared with its adjacent normal ones (2.0416 ± 0.2324) (Fig. 1; Table 3). One way ANOVAs confirmed the significant negative association between down expression of the *BRCA1* gene with Ki-67 expression status ($P_{value} \leq 0.001$).

Mean expression in patients with >35% Ki67 was 0.0640 ± 0.0069 in comparison with group with <15% ki67 (0.2725 ± 0.0303), group with 15–25% Ki67 (0.2711 ± 0.0351), and 25–35% Ki67 (0.2159 ± 0.0127) (S2) (Table 3).

RAD51 gene expression analysis in normal and tumor tissues

RAD51 was markedly down regulated by 9.9 fold in tumor breast samples (0.2013 ± 0.030) in comparison with corresponding adjacent normal tissues (1.8113 ± 0.2093) (Fig. 1; Table 4). Moreover, there was a significant association between down expression of *RAD51* and lymph node involvement ($P_{value} < 0.01$). *RAD51* gene expression level in group with more than 10 lymph node involvement was 0.1088 ± 0.0154 when compared with group with 4–9 lymph node involvement (0.1940 ± 0.0435), 1–3 lymph node involvement (0.2482 ± 0.0230) and without any lymph node involvement (0.2509 ± 0.0217) (Fig. 3a; Table 4). In addition, there was a significant association between PR (Progesterone Receptor) status ($P_{value} \leq 0.01$).

Table 2 *ATM* expression levels and clinicopathological and paraclinicalparameters in breast cancer sample

Clinicopathological features	*N* (%)	*ATM* relevant expression (mean ± SEM)	*P*	Clinicopathological features	*N* (%)	*ATM* relevant expression (mean ± SEM)	*P*
Tissues				Classification			
Breast tumor	50 (50)	0.1709 ± 0.0276		Yes	30 (60)	0.1579 ± 0.0253	
Adjacent normal	50 (50)	1.2240 ± 0.1386	0.0001	No	15 (30)	0.1834 ± 0.0268	0.389
Age				Malignancy			
<45	14 (28)	0.2066 ± 0.0425		Yes	15 (30)	0.0584 ± 0.0069	0.001
45–55	16 (32)	0.1313 ± 0.0333	0.176	No	26 (52)	0.2890 ± 0.0406	
>55	18 (36)	0.1726 ± 0.0217		Ki67 (%)			
Pathological grade				0–15	17 (34)	0.1497 ± 0.0139	
Grade I	12 (24)	0.1328 ± 0.0140		16–25	8 (16)	0.2344 ± 0.0234	0.198
Grade II	23 (46)	0.2154 ± 0.0251	0.389	26–35	8 (16)	0.1527 ± 0.0321	
Grade III	12 (24)	0.1691 ± 0.0246		> 35	12 (24)	0.1451 ± 0.0220	
Pathological types				Breast cancer Subtypes			
Invasive lobular carcinoma	9 (18)	0.1168 ± 0.0349		ER+/PR+/HER2+	14 (28)	0.2292 ± 0.0367	0.279
Invasive ductal carcinoma	27 (54)	0.1905 ± 0.0286	0.444	ER−/PR−/HER2−	9 (18)	0.1256 ± 0.0151	
Others	10 (20)	0.2048 ± 0.0337		ER+/PR+/HER2−	18 (36)	0.2210 ± 0.0392	
Auxiliary lymph node metastasis				ER−/PR−/HER2+	7 (14)	0.1057 ± 0.0036	
Yes	30 (60)	0.1293 ± 0.0247	0.457	ER			
No	18 (36)	0.2246 ± 0.0434		Positive	31 (62)	0.2496 ± 0.0347	0.004
Lymph node Involvement				Negative	17 (34)	0.1046 ± 0.0130	
N0	15 (30)	0.1454 ± 0.0262		PR			
N1–3	14 (28)	0.2384 ± 0.0381		Positive	29 (58)	0.2507 ± 0.0237	0.042
N4–9	7 (14)	0.1155 ± 0.0231	0.095	Negative	19 (38)	0.1078 ± 0.229	
>10	11 (22)	0.1814 ± 0.0198		HER2			
Tumor size				Positive	19 (38)	0.1804 ± 0.0318	0.193
<3 cM	30 (60)	0.1987 ± 0.0263	0.337	Negative	29 (56)	0.1595 ± 0.0342	
≥3 cM	16 (32)	0.1417 ± 0.0266		Necrosis			
Vascular invasion				Yes	28 (56)	0.0515 ± 0.0040	0.044
Yes	18 (36)	0.0610 ± 0.0085	0.007	No	16 (32)	0.2794 ± 0.0184	
No	24 (48)	0.3004 ± 0.0479		TNM Stage			
Perineural invasion				I	11 (24)	0.1045 ± 0.0209	
Yes	18 (36)	0.1149 ± 0.0330	0.037	II	26 (54)	0.1982 ± 0.0210	0.032
No	25 (50)	0.2250 ± 0.0454		III	10 (22)	0.2081 ± 0.0366	
Nerve invasion							
Yes	13 (26)	0.1205 ± 0.0205					
No	32 (64)	0.2260 ± 0.0355	0.068				

$P_{value} < 0.05$ was considered statistically significant

Mean expression in patients with PR positive was (0.1042 ± 0.0120) when compared with PR negative group (0.2967 ± 0.0378) (Fig. 3b; Table 4). In addition, there was a significant association between low expression of *RAD51* and a patient's age ($P_{value} \leq 0.001$). Patients with mean age >55 demonstrated the lowest expression (0.1213 ± 0.0247) in comparison with mean age 45–55 (0.1960 ± 0.0295) and mean age under the 45 years old (0.2944 ± 0.0253) (Fig. 3c; Table 4). There was a significant association between down expression of *RAD51* gene

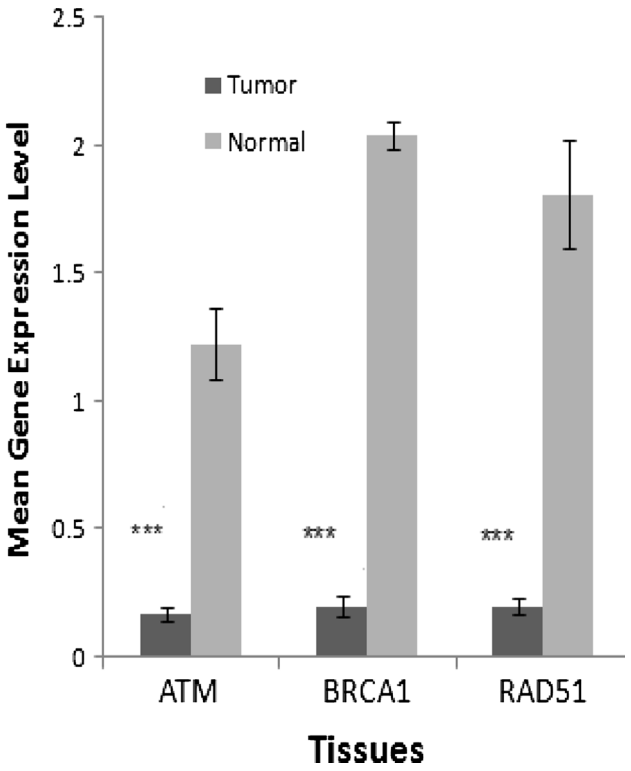

Fig. 1 Real-time PCR analysis of *ATM, BRCA1* and *RAD51* expression in breast cancer tumors. Using the $2^{-\Delta\Delta CT}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (*βeta actin*) and relative to normal control (adjacent normal tissue). ***$P_{value} < 0.001$

and pathological tumor grade. ($P_{value} < 0.05$). Mean expression in grade III tumor was 0.1252 ± 0.0324 in comparison with grade II (0.1910 ± 0.0208) and grade I (0.2893 ± 0.0491). (Figure 3d; Table 4).

There was a significant association between auxiliary lymph node metastasis and decreased expression level of RAD51. ($P \leq 0.01$). Mean expression in group that showed metastasis was 0.1373 ± 0.0116 and in group without metastasis was 0.2763 ± 0.0379. (Figure 3e; Table 4).

Correlation among ATM, BRCA1 and RAD51 expression levels in breast cancer patients

The $2^{-\Delta\Delta CT}$ results for *ATM*, *BRCA1* and *RAD51* in tumor tissues were statistically analyzed for each two gene (*ATM/BRCA1*, *ATM/RAD51* and *RAD51/BRCA1*) by Spearman's correlation test. The results indicated there was a significant positive correlation among *ATM/BRCA1* ($r = 0.641$, $P_{value} < 0.0001$), *BRCA1/RAD51* ($r = 0.764$, $P_{value} < 0.0001$) and ATM/RAD51 ($r = 0.619$, $P_{value} < 0.0001$) expression levels (Supplementary data, S3).

Correlation between ATM, BRCA1, and RAD51 expression and progress-free survival (PFS)

Clinical outcome of only 42 patients were available. Among them, only one passed away about 3 months after initial diagnosis. Two had metastasis: one to liver, one to lung. The rest are alive with no sign of recurrence of the disease or metastasis. Although, the expression levels of these three genes were low in these three patients, the number of the patients is too low to come to any conclusion regarding the correlation between the expression of these genes and PFS.

Discussion

Repair of DSBs is necessary to ensure genome integrity and cell viability [10]. ATM seems to be crucial as primary DSB sensor proteins. Activation of these proteins leads to posttranslational modification of downstream mediators, e.g. BRCA1. The mediators then reinforce the signal and trigger a signaling pathway that activates effectors proteins such as RAD51, P53 and checkpoint proteins which carry out cell cycle regulation, DNA repair and apoptosis [5].

Although some published reports indicated down expression of *ATM* in breast cancer [11, 12], some recent studies reported controversial results. Much higher expression level of *ATM* in ER-negative breast cancer was reported in one research [13]. In another study, it showed *ATM* expression is aberrantly reduced or lost in ER/PR/HER-2 negative breast cancers [12]. In two different previous research, no associations were found between low levels of ATM and different subclasses of breast cancer [14]. In addition, overexpression of *ATM* has been shown in myoepithelial carcinoma, prostate cancer and nasopharyngeal carcinoma [15–17]. Moreover, the association between *ATM* deregulation and prognostic factors in prognosis of breast cancer patients is limited.

In this study, our result showed decreased expression of *ATM* transcripts by 7.1 fold in tumor tissues in comparison with its normal adjacent ones ($P_{value} \leq 0.0001$). For the first time, we found a significant association between down expression of the *ATM* gene with vascular invasion ($P_{value} < 0.01$), perineural invasion ($P_{value} < 0.05$ and malignancy ($P_{value} \leq 0.001$) that indicate down expression of *ATM* correlates with aggressive behavior of tumors. In addition, for the first time a significant association has been found between *ATM* down-expression and tumor stage. Reduced expression of this gene would be due to LOH[2] [18, 19], aberrant methylation of promoter [2, 20] and post

[2] Loss of Heterozygosity.

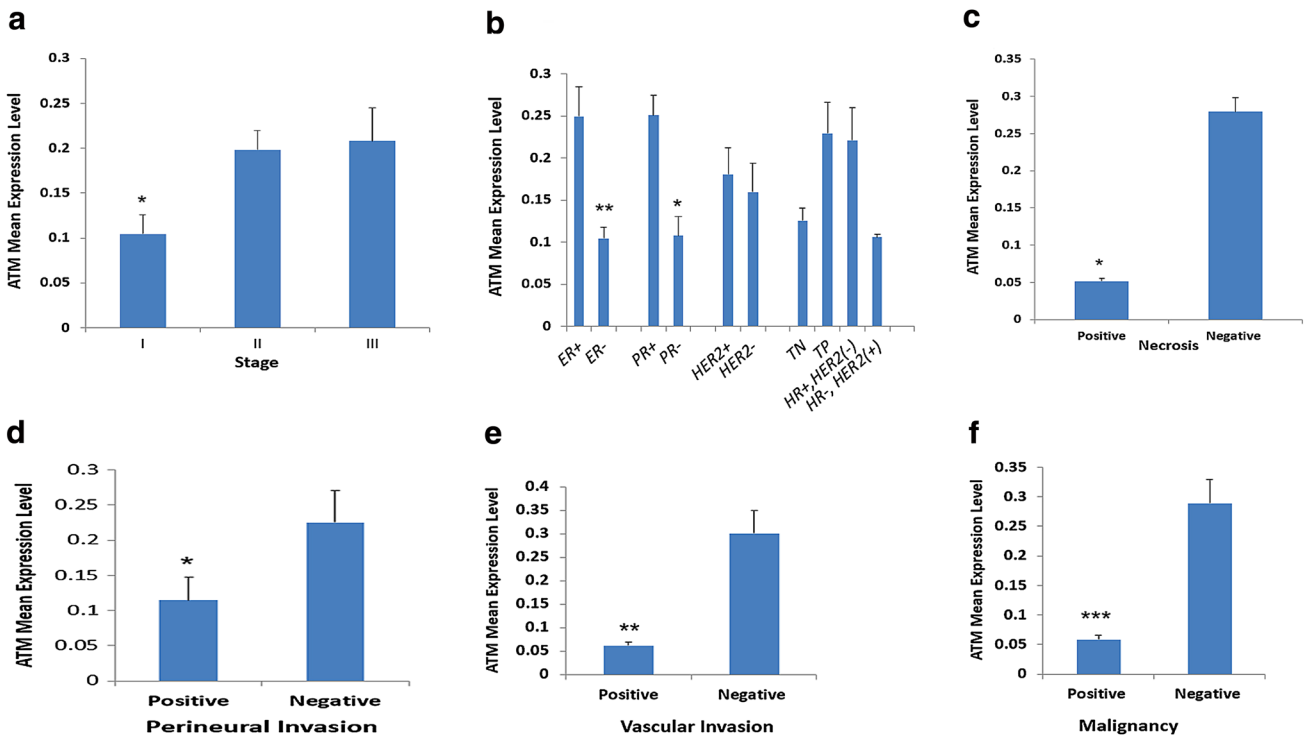

Fig. 2 *ATM* mean expression and clinicopathological factors. **a** *ATM* mean expression level and tumor stage. Samples were grouped according to pathological reports, **b** *ATM* mean expression in breast cancer subtypes. Patients were grouped considering IHC studies commonly used in clinical practice. Results are expressed as fold number decrease versus control (adjacent normal tissues). *ER* estrogen receptors, PR progesterone receptors, HER2 human epidermal growth factor 2, *TN* triple negative (ER−, PR−, and HER2−), *TP* triple positive (ER+, PR+, and HER2+). HR (hormone receptors: estrogen/progesterone receptors). $*P_{\text{value}} < 0.05$, $**P_{\text{value}} < 0.01$. **c** *ATM* mean expression level and Tumor Necrosis. $*P_{\text{value}} < 0.05$. **d** *ATM* mean Expression level and perineural invasion. $*P_{\text{value}} < 0.05$. **e** *ATM* mean expression level and vascular invasion. $**P_{\text{value}} < 0.01$. **f** *ATM* mean expression level and malignancy. $***P_{\text{value}} = 0.001$

transcriptional dysregulation such as overexpression of regulating miRNA [11].

Genetic analyses indicated that *BRCA1* is critical for HR pathway as well as the subnuclear assembly of *RAD51* after DNA damage [21]. We have found a significant reduction in the expression of *BRCA1* by 30 fold in breast tumors ($P_{\text{value}} \leq 0.001$). That is in line with other previous published report [22]. We found a significant association between down expression of the *BRCA1* gene and higher expression of Ki-67 ($P \leq 0.001$). Patients in group 4 (>35% proliferation rate) showed significant down expression, corroborating that *BRCA1* deregulations plays a role in cell proliferation. That is in agreement with a previous paper which found a negative correlation between the expression of *BRCA1* and Ki-67 [23].

Multiple mechanisms underlying inactivation and reduced expression of *BRCA1* include (a) loss of heterozygosity (LOH) [2, 8] (b) methylation of the *BRCA1* promoter region [24] (c) overexpression of some microRNA (miR-146a, miR-146b and miR-342 [24, 25]. (d) Mutations in transcription factors regulating the *BRCA1* promoter (2). (e) Alterations in the signaling pathways upstream of the transcription factors [2].

RAD51 is a crucial recombinase, often dysregulated in tumors [26]. While several studies have reported overexpression of *RAD51* in cell lines and tumors of different origins [26, 27] one study indicated down-regulation of *RAD51* expression in different cancer cell lines grown under chronic hypoxic conditions [28]. The exact cause of overexpression is not clarified. However, it has been suggested *TP53* deletions and some *TP53* point mutations up regulate the expression of *RAD51* [27, 28].

Although just one clinic research showed reduced expression of RAD51 in 30% of breast carcinoma at protein level, for the first time we are able to report down expression of *RAD51* in breast cancer tissues at the mRNA level. The change rate was significant by 9.9 fold in tumor samples when compared with its adjacent normal ones. Our results are in contrast with two other published reports in which overexpression of *RAD51* was observed. In one of these reports, patients were *P53* mutation positive and were exposed to neoadjuvant chemotherapy before surgery [5, 29], two options that are suggested to underlie *RAD51* overexpression. Patients in the other research have not above mentioned two options [30]. LOH is one of the mechanisms that leads to down expression of *RAD51* in

Table 3 *BRCA1* mean expression levels and clinicopathological and paraclinical parameters of breast cancer samples

Clinicopathological features	*N* (%)	*BRCA1* relative expression (mean ± SEM)	*P*	Clinicopathological features	*N* (%)	*BRCA1* relative expression (mean ± SEM)	*P*
Tissues				Classification			
Breast tumor	50 (50)	0.1979 ± 0.0458	0.001	Yes	30 (60)	0.1715 ± 0.0303	
Adjacent normal	50 (50)	2.0416 ± 0.2324		No	15 (30)	0.2297 ± 0.0365	0.278
Age				Malignancy			
<45	14 (28)	0.2317 ± 0.0223	0.465	Yes	15 (30)	0.2655 ± 0.034	
45–55	16 (32)	0.2003 ± 0.0290		No	26 (52)	0.1324 ± 0.0239	0.238
>55	18 (36)	0.1621 ± 0.0246		Ki67 (%)			
Pathological grade				0–15	17 (34)	0.2725 ± 0.0303	
Grade I	12 (24)	0.1831 ± 0.0253		15–25	8 (16)	0.2711 ± 0.0351	0.001
Grade II	23 (46)	0.1951 ± 0.0204	0.415	26–35	8 (16)	0.2159 ± 0.0127	
Grade III	12 (24)	0.2001 ± 0.0440		>35	12 (24)	0.0640 ± 0.0069	
Pathological types				Breast cancer Subtypes			
Invasive lobular carcinoma	9 (18)	0.2638 ± 0.0315		ER+/PR+/HER2+	14 (28)	0.1504 ± 0.0229	
Invasive ductal carcinoma	27 (54)	0.1573 ± 0.0228	0.484	Triple Negative	9 (18)	0.2406 ± 0.0178	0.217
Others	10 (20)	0.1741 ± 0.0349		ER+/PR+/HER2−	18 (36)	0.1804 ± 0.0324	
Auxiliary lymph node metastasis				ER−/PR−/HER2+	7 (14)	0.2169 ± 0.0110	
Yes	30 (60)	0.1812 ± 0.0205		ER			
No	18 (36)	0.2116 ± 0.0208	0.659	Positive	31 (62)	0.1830 ± 0.0258	
Lymph node Involvement				Negative	17 (34)	0.2115 ± 0.0286	
N0	15 (30)	0.2178 ± 0.0285		PR			
N1–3	14 (28)	0.2281 ± 0.0230		Positive	29 (58)	0.1261 ± 0.0213	0.399
N4–9	7 (14)	0.1964 ± 0.0361	0.281	Negative	19 (38)	0.2639 ± 0.0327	
>10	11 (22)	0.1489 ± 0.0261		HER2			
Tumor size				Positive	19 (38)	0.1652 ± 0.0386	
<3 cM	30 (60)	0.2147 ± 0.0255		Negative	29 (58)	0.2259 ± 0.0247	0.408
≥3 cM	16 (32)	0.1780 ± 0.0266	0.181	Necrosis			
Vascular invasion				Yes	30 (60)	0.1574 ± 0.0184	0.098
Yes	18 (36)	0.1596 ± 0.0262		No	18 (36)	0.2360 ± 0.0123	
No	24 (48)	0.2312 ± 0.0315	0.135				
Perineural invasion				TNM Stage			
Yes	18 (36)	0.1574 ± 0.0412		I	11 (24)	0.1902 ± 0.0246	
No	25 (50)	0.2403 ± 0.0290	0.558	II	26 (54)	0.2438 ± 0.0240	
Nerve invasion				III	10 (22)	0.1566 ± 0.0261	0.073
Yes	15 (30)	0.1026 ± 0.0277	0.376				
No	32 (64)	0.2953 ± 0.0244					

$P_{value} < 0.05$ was considered statistically significant

breast cancer [2]. Moreover, overexpression miRNA could lead to *RAD51* down expression [31]. The association between *RAD51* gene expression level and clinicopathological factors is limited. In this study, a significant association was clarified between down expression of *RAD51* and clinical and preclinical parameters, such as lymph nodes involvement, auxiliary lymph node metastasis, age, grade and PR status. In this research, samples with

Table 4 *RAD51* mean expression levels and clinicopathological and paraclinical parameters of breast cancer samples

Clinicopathological features	*N* (%)	*RAD51* relative expression (mean ± SEM)	*P*	Clinicopathological features	*N* (%)	*RAD51* relative expression (mean ± SEM)	*P*
Tissues				Classification			
Breast tumor	50	0.2013 ± 0.030		Yes	30 (60)	0.1860 ± 0.0226	0.067
Adjacent normal	50	1.8113 ± 0.2093	0.0001	No	15 (30)	0.2144 ± 0.0419	
Age				Malignancy			
<45	14 (28)	0.2944 ± 0.0253		Yes	15 (30)	0.1501 ± 0.0160	0.202
45–55	16 (32)	0.1960 ± 0.0295	0.001	No	26 (52)	0.2549 ± 0.03873	
>55	18 (36)	0.1213 ± 0.0247		Ki67			
Pathological grade				0–15%	17 (34)	0.2205 ± 0.0392	
Grade I	12 (24)	0.2893 ± 0.0491		16–25	8 (16)	0.2253 ± 0.0288	0.089
Grade II	23 (46)	0.1910 ± 0.0208	0.027	26–35	8 (16)	0.2503 ± 0.0408	
Grade III	12 (24)	0.1252 ± 0.0324		>35	12 (24)	0.1124 ± 0.0047	
Pathological types				Breast cancer Subtypes			
Invasive lobular carcinoma	9 (18)	0.2400 ± 0.0453		ER+/PR+/HER2+	14 (28)	0.1472 ± 0.0334	
Invasive ductal carcinoma	27 (54)	0.1729 ± 0.0273	0.493	Triple negative	9 (18)	0.2717 ± 0.0287	0.314
Others	10 (20)	0.1906 ± 0.0257		ER+/PR+/HER2−	18 (36)	0.1731 ± 0.0274	
Auxiliary lymph node metastasis				ER−/PR−/HER2+	7 (14)	0.2171 ± 0.0205	
Yes	30 (60)	0.1373 ± 0.0116		ER			
No	18 (36)	0.2763 ± 0.0379	0.01	Positive	31 (62)	0.1220 ± 0.018	
Lymph node Involvement				Negative	17 (34)	0.2934 ± 0.0379	0.210
N0	15 (30)	0.2509 ± 0.0217		PR			
N1–3	14 (28)	0.2482 ± 0.0230		Positive	29 (58)	0.1042 ± 0.0120	0.016
N4–9	7 (14)	0.1940 ± 0.0435	0.004	Negative	19 (38)	0.2967 ± 0.0378	
>10	11 (22)	0.1088 ± 0.0154		HER2			
Tumor size				Positive	19 (38)	0.1856 ± 0.0369	
< 3 cM	30 (60)	0.2643 ± 0.0214		Negative	29 (58)	0.2184 ± 0.0282	0.325
≥ 3 cM	16 (32)	0.1457 ± 0.0273	0.441	Necrosis			
Vascular invasion				Yes	28 (56)	0.1643 ± 0.0324	
Yes	18 (36)	0.1700 ± 0.0279		No	16 (32)	0.2352 ± 0.0410	
No	24 (48)	0.2264 ± 0.0362	0.184	TNM Stage			0.593
Perineural invasion				I	11 (24)	0.1804 ± 0.0365	
Yes	18 (36)	0.1820 ± 0.0378	0.448	II	26 (54)	0.1200 ± 0.0197	
No	25 (50)	0.2216 ± 0.0254		III	10 (22)	0.2103 ± 0.0468	0.098
Nerve invasion							
Yes	13 (26)	3.513 ± 0.4058	0.161				
No	32 (64)	4.987 ± 0.7546					

$P_{value} < 0.05$ was considered statistically significant

PR positive showed significant decrease in comparison with samples with PR negative.

Finally, our data indicated a significant positive correlation among *ATM/BRCA1* ($r = 0.641$, $P < 0.0001$), *BRCA1/RAD51* ($r = 0.764$, $P < 0.0001$) and ATM/RAD51 ($r = 0.619$, $P < 0.0001$) expression levels.

It's ostensible that targeted consideration of gene expression variation in specific cell pathways can exhibit

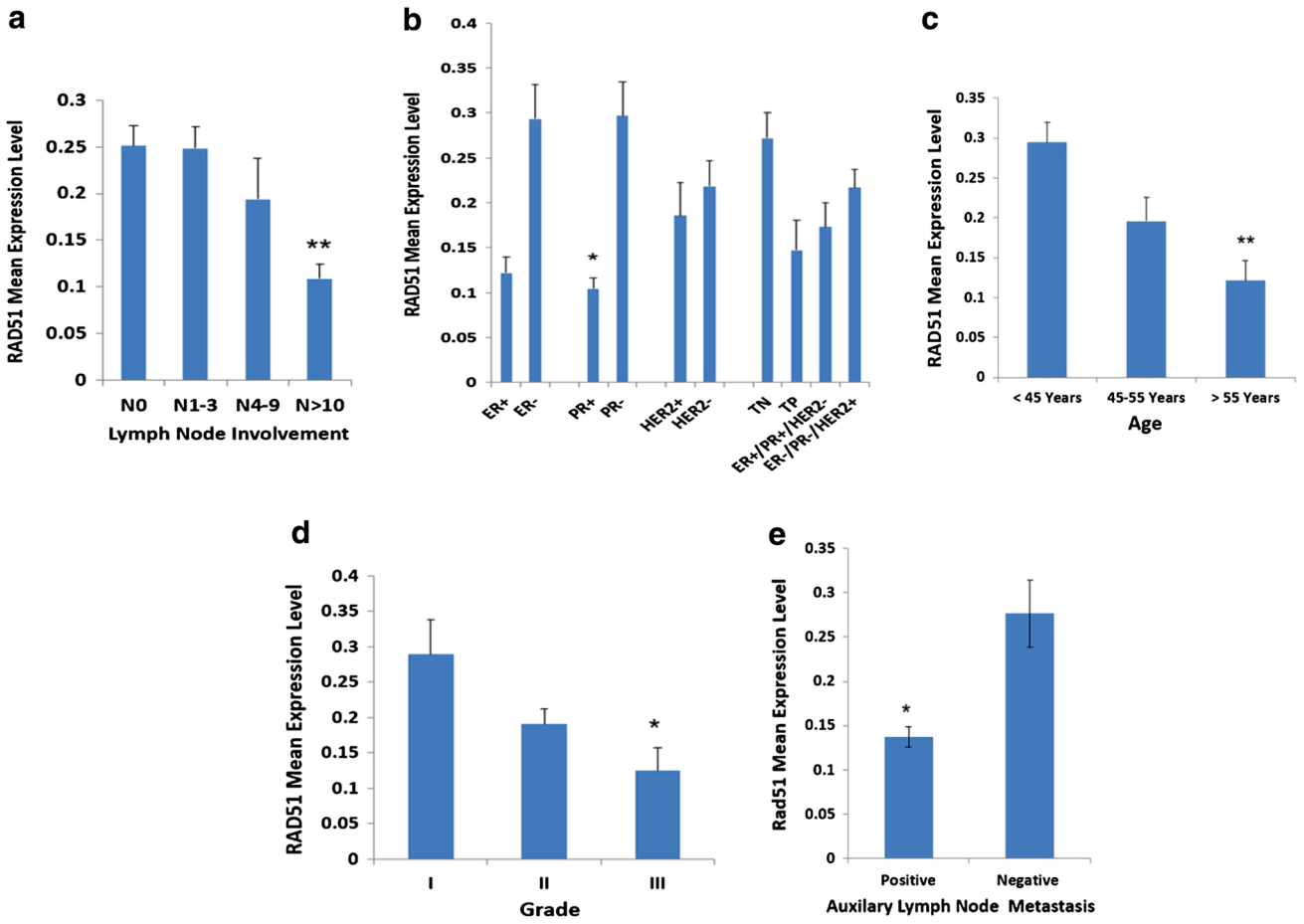

Fig. 3 *RAD51* mean expression and clinicopathological factors. **a** *RAD51* mean expression level and lymph node involvement. $^{**}P < 0.01$. **b** *RAD51* mean expression in breast cancer subtypes. Patients were grouped considering IHC studies commonly used in clinical practice. Results are expressed as fold number decrease versus control (adjacent normal tissues). ER estrogen receptors, PR progesterone receptors, HER2 human epidermal growth factor2, *TN* triple negative (ER−, PR−, HER2−). *TP* triple positive (ER+, PR+, HER2+). HR (hormone receptors: estrogen/progesterone receptors).$^{*}P < 0.05$. c) *RAD51* mean expression level and age. $^{**}P < 0.01$. **d** *RAD51* mean expression level and tumor grade. Patients were grouped according to pathological reports. $^{*}P < 0.05$. **e** *RAD51* mean expression level and auxiliary lymph node metastasis. $^{*}P_{\text{value}} < 0.05$

some unknown biological properties that are not appeared in single gene changes. Although some studies reported down expression of *ATM*, *BRCA1* [6, 8, 24]. However, albeit the major role of *ATM* in HR pathway there is no clinical research examining the correlation of expression changes of *ATM* with *BRCA1* and *RAD51* expression. We found significant correlation between down expression of these genes analyzed via SPSS software version 22. These results supported the hypothesis that upstream genes can regulate other downstream genes in the same pathway or genes in the same pathway, are regulated by same regulator through related mechanisms in a coordinated way.

(I) Downstream proteins are regulated by upstream ones. There are some interactions between *BRCA1*, *ATM* and *RAD51* in HR repair such that *ATM* regulates *BRCA1* [4] and *BRCA1* regulates *RAD51* [21]. *ATM* acts as the upstream sensor and in mammalian cells it is necessary for the initiation of a signaling pathway. Following DSB formation, ATM phosphorylates BRCA1 and TP53 to promote DSB repair and cell cycle regulation [4]. BRCA1 is a substrate of ATM in vitro and in vivo. It has been revealed that a part of the cellular response to DNA damage *BRCA1* is regulated by an ATM dependent mechanism [4]. On the other hand, the major mechanism underlying down expression of *BRCA1* in breast cancer is not clear. Methylation appears to be a significant factor in *BRCA1* regulation only in a small proportion of breast tumors [32]. Since, LOH associated with low expression of *BRCA1* in the minority of cases, it is an inadequate explanation as a cause for reduced expression of *BRCA1* in breast carcinoma [33]. Previous research confirmed that BRCA1 regulate and activate RAD51 [21]. Therefore, down expression of *ATM* can be a cause for low expression of *BRCA1* and *RAD51* genes that act in concert downstream

of *ATM* in the same pathway in breast cancer. In this hypothesis, down-expression *ATM* would underlie down expression of *BRCA1* and down expression of the last gene leads to down expression of *RAD51*.

(II) Expression of these genes is regulated by related mechanisms. A same transcription factor regulates transcription of these genes. For example, E2F1 can elevate the expression of genes *BRCA1* [34], *RAD51* [28, 35] and *ATM* [36]. In addition, Epithermal growth factor receptor (EGFR) promotes DSB repair by interacting with ATM, RAD51 and BRCA1 [37] and regulate expression of these genes. Moreover, polo- like kinase 1 regulates transcription of both *BRCA1* and *RAD51* [38]. This raises the possibility co regulation of these genes, with the same transcription factors, can lead to down expression of these three genes in breast cancer. So up and down expression of regulators can underlie the expression all of target downstream genes. For future perspective it would very valuable to examine the whole tumorigenic pathway to achieve better insight into the molecular changes involved in breast cancer development and progression.

It has to be noted that in this study we only investigated the gene expression at mRNAs levels and not protein levels. This transcription level data can suggest that these proteins are probably present more in normal breast tissues compared to breast tumoral tissues roughly and at what level to expect to see these proteins. As a future work one might investigate the expression level of these genes at their protein levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human rights statement and informed consent All procedures followed were approved by the local ethical standards of National Institute of Genetic Engineering and Biotechnology (NIGEB) with IR.NIGEB.EC.1395.5.6.B approval number. Written informed consent was obtained from each patient who participated in this study prior to sample collection.

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