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Original article

PAX5α and *PAX5β* mRNA expression in breast Cancer: Relation to serum P53 and MMP2

Manal Basyouni Ahmed [a,b,*], Enas Samir Nabih [b], Muneera Al-Sheeha [c]

[a] *Medical Biochemistry Department, Faculty of Medicine, Qassim University, Saudi Arabia*
[b] *Medical Biochemistry Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[c] *Obs/Gyn/Medicine, Faculty of Medicine, Qassim University, Saudi Arabia*

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ABSTRACT

Background: Many studies evaluated the role of paired box gene 5 (PAX5) in breast cancer. However, few investigated *PAX5α* and *PAX5β* isoforms individually. *Objective:* The aim of the present study is to evaluate mRNA expression of PAX5α and PAX5β in breast cancer and assessing their underlying pathological roles through investigating their correlations to matrix metalloproteinase2 (MMP2), p53 and cancer antigen 15-3 (CA15-3). *Methods:* Evaluation of *PAX5α* and *PAX5β* RNA expression in breast tissue samples was done by real-time PCR, serum MMP2 and p53 by ELISA and CA15-3 by chemiluminescent immunoassay in 55 infiltrating ductal carcinoma patients, 25 fibroadenoma patients and 15 control females who underwent plastic breast surgery. *Results: PAX5α* RNA was reduced while *PAX5β* RNA was elevated in breast cancer patients compared to other groups ($p < 0.01$). The best cutoff value of *PAX5α* was $\leqslant 1.15$ with 78.2% sensitivity and 90% specificity and for PAX5β it was $\geqslant 2.01$ with 83.6% sensitivity and 82.5% specificity. The RNA expression of *PAX5β* was higher in advanced grades and stages ($p < 0.01$), and significantly correlated to p53 (r: 0.51, $p < 0.01$), and CA15-3 (r: 0.45, $p < 0.01$). Moreover, *PAX5α* was negatively correlated to *PAX5β* (r: −0.42, $p < 0.01$), p53 (r: −0.53, $p < 0.01$) and MMP2 (r: −0.22, $p < 0.05$). *Conclusions:* The downregulation of *PAX5α* with upregulation of *PAX5β* RNA expressions could highlight the possible oncogenic role of *PAX5* as a transcriptional factor in tumorigenesis and progression of breast cancer.
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1. Introduction

Breast cancer is the most common cancer among women and the fifth leading cause of cancer deaths worldwide. It represents 14.7% of all cancer deaths in women [1]. However, mortality from this disease can be reduced greatly through the understanding of the molecular alterations that contribute to disease initiation and progression [2].

Paired box (*PAX*) proteins function as important nuclear transcription factors for cellular differentiation, migration, and proliferation. Most of these genes are silent in the adult stage and their expressions were reported to play important roles in conferring growth and survival of cancer cells [3]. *PAX5* is expressed during B lymphopoiesis and is considered the regulatory gene of the B-cell gene expression until plasma cell differentiation [4]. *PAX5* gene was also expressed in the new forming central nervous system and adult mouse testis [5], as well as brain tissue and some tissues of the male and female genital tracts in adult human [6]. *PAX5* expression was extended beyond B-cell cancers, to affect other carcinogenic events in tissues of non-lymphoid origin [7].

Many studies reported the carcinogenic role of *PAX5* by promoting cell growth and survival. Cozma et al. [8] found that *PAX5* may contribute to oncogenesis through the STAT signaling pathway and/or by the direct inhibition of p53 expression in murine lymphomas. Additionally, *PAX5* knockdown in human lymphoma might have a negative effect on cell expansion. Furthermore, *PAX5* through its downstream transcription factor c-Met; a receptor tyrosine kinase was involved in cell motility and angiogenesis in small-cell lung cancer [9]. Among the various downstream signals of *Pax5* target genes are many cancer-associated proteins, such as p53, human-telomerase reverse transcriptase (hTERT), and mesenchymal markers [i.e., matrix metalloproteinase 2 (MMP-2), vimentin, and tyrosine-protein kinase Met (c-Met)] [7]. Indeed, other studies suggested that PAX5 had opposing effects on proliferative activity and negative effects on cancer migration and motility. Li et al. [10] revealed that the anti-tumorigenic function of *PAX5* in primary gastric tumors was mediated by its directly down

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* Corresponding author at: Faculty of Medicine, Ain Shams University, P.O. 38, Cairo, Egypt.
E-mail address: manal_basiony@yahoo.com (M.B. Ahmed).

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regulation of many oncogenes. Whereas increased *PAX5* expression has been reported in different malignancies, its precise role in cancer remains unclear, and conflicting evidence exists suggestive of both tumor-promoting and tumor suppressor functions [11].

The inconsistent functions of *PAX5* possibly resulted from its isoforms that are expressed differently in various types of cells [7]. *PAX5* gene is located on chromosome 9p13, that is associated frequently with chromosomal translocations and contains two distinct promoters resulting in two alternative 5′ exons (α and β) that are spliced to common coding sequences of exons 2–10 [12]. Previous reports have contributed to the functional characterization of *PAX5* at the molecular and cellular level, however few have discriminated between α and β isoforms individually. Therefore, this study was conducted to investigate the possible role of *PAX5α* and *PAX5β* isomers in the pathogenesis of breast cancer by correlating their expressions to their downstream effectors as p53, MMP2, a tumor marker as CA15-3, and to the different clinicopathological factors in breast cancer patients.

2. Subjects and methods

2.1. Patients' database

This prospective study was carried out; from Jan. 2014 to Nov. 2015; on 95 Egyptian females selected from General Surgery Department, Ain Shams University Hospitals, Egypt. Tumors were staged according to tumor node metastasis (TNM) classification of the American Joint Committee on (AJCC) [13] and graded by the Nottingham grading system [14]. The investigated participates were divided into; ***malignant group***: included 55 patients with infiltrating ductal breast carcinoma of median age 45 years (range 37–56 years), ***benign group***: included 25 patients with fibroadenoma of median age 46 years (rang from 34 to 58 years), and ***control group***: included 15 normal volunteers' females who underwent plastic cosmetic breast surgery of median age 42 years (rang from 35 to 45 years). Reports of pathology, estrogen, and progesterone hormonal status were obtained from hospital records. 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. An informed consent: "Informed consent was obtained from all individual participants included in the study.

2.2. Samples collection and processing

Surgically removed breast tissues were obtained prior to any initial chemotherapy or hormonal therapy. Breast samples were washed in ice-cold saline then kept frozen at −80 °C until used in the determination of *PAX5α* and *PAX5β* expression by real-time PCR. Five milliliters of blood were collected from each participant under complete aseptic conditions into tubes without anticoagulant and centrifuged at 14,000 revolutions per minute (rpm) for 10 min. Serum samples were kept and frozen at −80 °C until used for the quantitative determination of p53, MMP2 and CA15-3.

2.3. Evaluation of serum MMP2 and p53

Serum MMP2 levels (ng/ml) and p53 levels (pg/ml) were determined according to manufacturer's instructions using commercially available ELISA kit. MMP2 kit supplied by Quantikine, R&D Systems, Minneapolis, MN [15] and p53 kit supplied by Sigma–Aldrich Chemie GmbH, Steinheim, Germany [16].

2.4. Evaluation of serum CA 15-3

Serum CA 15-3 levels (U/ml) were determined by using chemiluminescent immunoassay by CA15-3 assay kits (Centocor, Malvern, Pa) method done on the architect System [17].

2.5. Real-time PCR

The isolation of total RNA from the breast tissues was done according to *Bird* [18], using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). The purity and concentration of RNA were determined by spectrophotometric measurement of absorbance at 260 and 280 nm [19]. cDNA was synthesized using Ready-To-GoYou-Prime First-Strand Beads (GE Healthcare) [20]. Complementary DNA (2 μg) was amplified in a 25-μL reaction containing 13 μL of iQSYBRGreenSupermix (Bio-Rad), 1 μL each of *PAX5α* primers (forward, 5′-CCTGTCCATTCCATCAAGTCCTG-3′; reverse, 5′-TTTTGCTGACACAACCATGGCTGAC-3′, GenBank: XM_011517896.1) or 1 μL each of *PAX5β* primers (forward, 5′-CCCGATGGAAATACACTGTAAGCAC-3′; reverse, 5′-TTTTGCTGACACAACCATGGCTGAC-3′, GenBank: XM_005251481.3) and 8 μL of nuclease-free water. The real-time PCRs were done at 95 °C for 3 min, 40 cycles of PCR were performed under the following conditions: 95 °C, 57 °C and 75 °C for 10 s, 30 s and 30 s respectively. The following primer sequences were used for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-TCAATGGAAATCCCATCACCA-3′; reverse, 5′-TGATTTTGGAGGGATCTCGCT-3′, GenBank: NM_001289746.1). Bio-Rad software was used to calculate threshold cycle (Ct) values for the target genes and for GAPDH. The expression values for the tumor samples are presented as fold expression in relation to the control sample; the actual values were calculated using the $2-\Delta\Delta Ct$ equation, where $\Delta\Delta Ct$ = [Ct Target – Ct GAPDH] (tumor sample) − [Ct Target – Ct GAPDH] (control sample). All samples were analyzed using the Rotor Gene real time PCR detection system (Qiagen, Hilden, Germany).

2.6. Statistical analysis

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 20, SPSS Inc., Chicago, IL). Parametric test, ANOVA (followed by Post Hoc test) or non-parametric Mann-Whitney U (to compare two groups) and Kruskal-Wallis tests (to compare three groups) were applied to compare the parameters statistically. Quantitative parameters were compared between groups by using Chi-square test. Moreover, Pearson's correlation coefficient was applied to Correlate between different variables. The parameters were also correlated with the clinicopathological factors using the non-parametric test Kruskal-Wallis ($\chi 2$) test. Statistical significance was set at a value of $p < 0.05$. Receiver operating characteristic (ROC) curve was used to discriminate positive from negative results. It determined the threshold value for optimal sensitivity and specificity, which was constructed by calculating the true positive fraction (sensitivity percent) and false positive fraction (100-specificity) of markers at several cutoff points.

3. Results

Clinicopathological factors of the studied groups were shown in the Table 1. The malignant group had a significantly lower expression of *PAX5α*, higher expression of *PAX5β* and higher serum levels of MMP2, p53 and CA 15-3 proteins compared to benign and control groups ($p < 0.01$). Moreover, the benign group had significantly lower PAX5α expression and higher p53 serum levels compared to the control group ($p < 0.01$), Table 2. The best cutoff values for the

Table 1
Clinicopathological factors of the study groups.

Clinicopathological factors	Normal No. (%)	Benign No. (%)	Malignant No. (%)	Statistics
Age				
Median (range)	42 (35–45)	46 (34–58)	45 (37–56)	–
Menopausal status				χ^2: 3.74
Premenopause	12 (80.0%)	13 (52%)	17 (30.9%)	p > 0.05
Postmenopause	3 (20.0%)	12 (48%)	38 (69.1%)	
Parity				
0	5 (33.3%)	4 (16%)	4 (7.2%)	χ^2: 9.1
1–2 children	6 (40%)	11 (44%)	18 (32.7%)	p > 0.05
⩾3 children	4 (26.7%)	10 (40%)	33 (60.1%)	
Grade				
Grade 1		–	7 (12.7%)	
Grade 2		–	33 (60.0%)	
Grade 3		–	15 (27.3%)	
Stages				
Stage I		–	5 (9.1%)	
Stage II		–	30 (54.5%)	
Stage III		–	20 (36.4%)	
Tumor size				
<2 cm			2 (3.6%)	
2–5 cm			31 (56.3%)	
>5 cm			22 (40.1%)	
+ve Estrogen R			39 (70.9%)	
+ve Progesterone R			42 (76.4%)	

p > 0.05 is non-significant.

Table 2
Expression levels of *PAX5α* and *PAX5β*, and serum levels of MMP2 and p53 in the studied groups.

Parameters	Normal	Benign	Malignant	p value
PAX5α				p1 < 0.01**
Mean ± SD	3.59 ± 1.44	2.04 ± 1.1	1.29 ± 0.66	p2 < 0.01**
Median	3.3	1.8^{p1*}	$1.01^{p1*\&\ p2*}$	p3 < 0.01**
PAX5β				p1 > 0.05
Mean ± SD	1.03 ± 0.06	1.59 ± 0.56	3.43 ± 1.55	p2 < 0.01**
Median	1.00	1.69	$3.04^{p1*\&\ p2*}$	p3 < 0.01**
MMP2 (ng/ml)				p1 > 0.05
Mean ± SD	5.1 ± 1.33	4.78 ± 1.44	5.97 ± 0.88	p2 < 0.01**
Median	4.2	4.5	$5.9^{p1*\&\ p2*}$	p3 < 0.01**
p53 (pg/ml)				p1 < 0.01**
Mean ± SD	68.8 ± 22.4	98.5 ± 6.3	111.2 ± 11.4	p2 < 0.01**
Median	66.2	100.4^{p1*}	$106.0^{p1*\&\ p2*}$	p3 < 0.01**
CA 15-3 (U/ml)				p1 > 0.05
Mean ± SD	5.7 ± 2.6	12.4 ± 6.22	25.8 ± 12.9	p2 < 0.01**
Median	4.2	7.5	$18.9^{p1*\&\ p2*}$	p3 < 0.01**

p1 = benign group versus healthy controls, p2 = malignant group versus benign group and p3 = malignant group versus healthy controls. Statistical comparisons were made using parametric test (median), ANOVA (followed by Post Hoc test).
[PAX5: paired box gene 5, MMP2: matrix metalloproteinase2, CA15-3: cancer antigen 15-3].
** $p < 0.01$ is highly significant. $p > 0.05$ is non-significant.

investigated markers were determined by ROC curve by considering benign and healthy normal groups as the non-malignant group. The best cutoff value for *PAX5α* was below 1.15 (78.2% sensitivity, 90% specificity, 0.868 Area Under ROC Curve (AUC), for PAX5β was above 2.01 (83.6% sensitivity, 82.5% specificity, 0.882 AUC), for serum MMP2 was above 5.0 ng/ml (92.7% sensitivity, 72.5% specificity, 0.75 AUC) and for serum p53 was above 101.2 pg/ml (74.5% sensitivity, 77.5% specificity, 0.8 AUC), Fig. 1. The positivity rates of all investigated parameters were higher in the malignant group compared to other groups (p < 0.01), Fig. 2. Assessment of the relation of *PAX5α* and *PAX5β* to different clinicopathological factors in our malignant patients revealed that the expression level of *PAX5β* was lower in grade1 and stage I (p < 0.01), Table 3. PAX5α showed significant negative correlations to *PAX5β* (p < 0.01), MMP2 (p < 0.05) and p53 (p < 0.01), while *PAX5β* was significantly positively correlated to p53 and CA15-3 (p < 0.01), Table 4.

4. Discussion

Many studies reported the conflict role of *PAX5* as a tumor promoting gene by activating cell proliferation and migration while others suggested it as a tumor opposing gene that favors epithelium differentiation. Few studies evaluated the expression of *PAX5α* and *β* variants in cancer although this might explain these seemingly conflicting functions exhibited by *PAX5* gene. Therefore, we conducted this study to investigate *PAX5α* and *PAX5β* expression in breast cancer and correlate their expression to MMP2 and p53 to shed light on the role of these variants in the molecular basis of breast cancer.

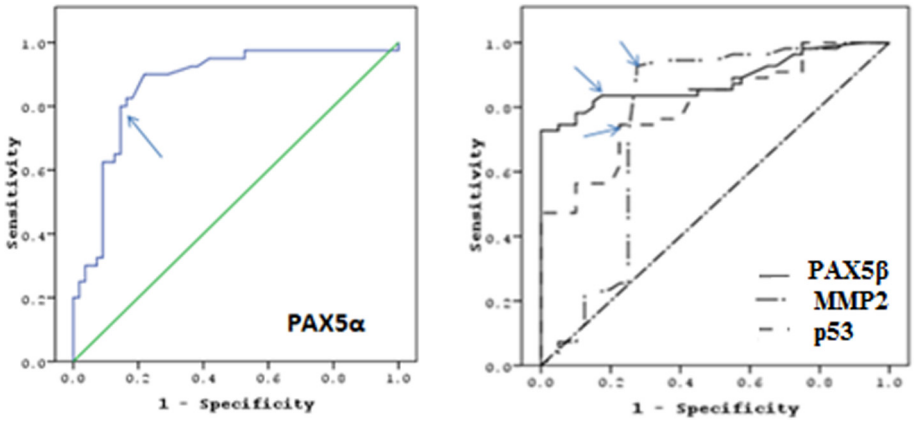

Fig. 1. ROC curve analysis for *PAX5α* (⩽1.15, AUC: 0.868), *PAX5β* (⩾2.01, AUC: 0.882), MMP2 (⩾5.0 ng/ml, AUC: 0.75) and p53 (⩾101.2 pg/ml, AUC: 0.8) to calculate the best cut-off point to discriminate between malignant and non-malignant groups, $p < 0.01$.

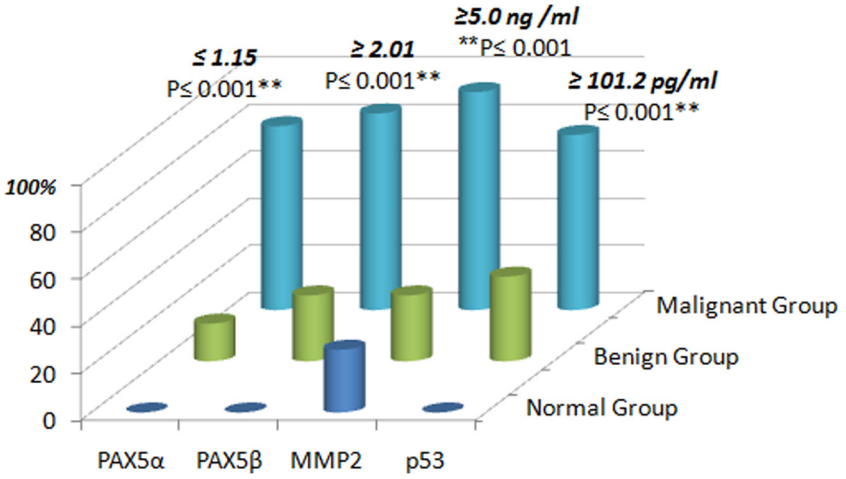

Fig. 2. Positivity rate of *PAX5α, PAX5β*, MMP2 and p53 in the malignant group compared to benign and control groups. $^{**}p < 0.01$ is highly significant.

Previously, we demonstrated that *PAX5* gene was nearly not expressed in normal and benign breast tissues and was significantly expressed in malignant tissues (94.0% sensitivity and 95.0% specificity, $p < 0.01$). Moreover, we found that the expression of *PAX5* and CD19 genes was positively correlated knowing that CD19 is implicated in the control of proliferation and differentiation [21]. In an attempt to continue our work, we evaluated the expression of *PAX5α* and *β* in breast tissue specimens. Our results showed that β isomer was significantly up-regulated with concomitant down-regulation of α isomer in the studied malignant breast tissues. O'Brien et al. [7] reported that the oncogenic transcription factor *PAX5* is an important developmental regulator that is implicated in the pathogenesis of several malignancies, however, they did not investigate *PAX5α* or *β* isoforms. Meanwhile, a gene expression microarray analysis revealed a 100-fold overexpression of *PAX5* in breast cancer cells that metastasized to lymph nodes compared to the primary tumor [22]. The inverse relationship between *PAX5α* and *β* isoforms detected in this study was in agreement with previous reports. Vidal et al. [23] reported a none significant difference in *PAX5* expression between normal and cancerous breast tissues and attributed this finding to the up and down differential expression of its isomers. Similarly, Robichaud et al. [24] explained the negative autoregulatory effect between 2 distinct promoters of *PAX5α* and *PAX5β* isoforms as by suppressing the expression of *PAX5β* resulted in upregulation of *PAX5α* expression level.

In an attempt to evaluate both isomers in breast cancer, we conducted ROC curve by considering the normal and benign group as the non-malignant group, *PAX5α* at a cutoff 0.15 showed 78.2% sensitivity and 90% specificity, while *PAX5β* at a cutoff 2.01 showed 83.6% sensitivity and 82.5% specificity. CA15-3 is one of the most widely used serum tumor biomarkers in the different molecular subtypes of breast cancer [25]. The additional significant correlation between CA15-3 and *β* isomer highlights *PAX5β* to modulate aggressiveness in breast cancer patients. Our result represents a novel finding as to our knowledge no studies are available regarding the previous correlation. Although Baumann Kubetzko et al. [11] explored a relationship between *PAX5* expression and the more malignant phenotype of neuroblastoma cell lines, yet they did not rely their evidence based on which isomer was the most prominent.

Among the *PAX5* target genes are many cancer-associated proteins, such as p53 and MMP-2 [7]. We correlated *PAX5α* and *β* expression to the serum levels of MMP2 and p53 in an attempt to clarify the downstream effectors of *PAX5* variants and to further understand their mechanistic actions in the pathogenesis of breast cancer. p53 was negatively correlated to *PAX5α* and positively correlated to *PAX5β*. However, this pro-apoptotic activity of *PAX5β* did not decrease tumorigenesis since its expression was correlated with aggressive tumor behavior of advanced grades and stages. This could be explained by p53 mutation which has been associated with more aggressive disease and worse survival of breast cancer patients [26]. Liu et al. [27] revealed that ectopic expression

Table 3
Relation between *PAX5α* and *PAX5β* expression and clinicopathological factors in the malignant group.

Clinicopathological factors	N-fold PAX5α mRNA expression		N-fold PAX5β mRNA expression	
	Mean Rank	Positivity rate ⩽ 1.15	Mean Rank	Positivity rate ⩾ 2.01
Menopausal status				
Premenopause	46.77	12/17 (70.6%)	47.89	13/17 (76.5%)
Postmenopause	48.63	31/38 (81.57%)	48.22	33/38 (86.8%)
Parity				
0	39.12	3/4 (75.0%)	32.46	2/4 (50.0%)
1–2 children	44.86	14/18 (77.8%)	45.93	15/18 (83.3%)
⩾3 children	52.80	26/33 (78.79%)	53.84	29/33 (87.87%)
Grade				
Grade 1	28.3	5/7 (71.4%)	13.1	2/7 (28.5%)
Grade 2	28.1	26/33 (78.7%)	26.6	30/33 (90.1%)
Grade 3	27.7	12/15 (80%)	38.0	14/15 (93.3%)
			χ^2: 12.17	χ^2: 17.8
			$^{**}p = 0.002$	$^{**}p = 0.001$
Stage				
Stage I	34.1	3/5 (60%)	9.7	1/5 (20%)
Stage II	27.8	24/30 (80%)	26.0	26/30 (86.6%)
Stage III	26.8	16/20 (80%)	35.55	19/20 (95.0%)
			χ^2: 11.4	χ^2: 16.8
			$^{**}p = 0.003$	$^{**}p = 0.001$
Estrogen receptor				
Negative	27.6	13/16 (81.3%)	24.80	12/16 (75.0%)
Positive	27.46	30/39 (76.9%)	28.54	34/39(87.17%)
Progesterone receptor				
Negative	26.25	10/13 (76.3%)	27.50	11/13 (84.6%)
Positive	27.86	33/42 (78.5%)	28.06	35/42 (83.3%)

[PAX5: paired box gene 5].
** $p < 0.01$ is highly significant.

Table 4
Correlation (r) between the investigated parameters.

Parameters	*PAX5α*		*PAX5β*		MMP2		pP53	
	r	p	r	p	r	p	r	p
CA 15-3	−0.040	0.772	0.450**	0.000	0.440**	0.001	0.390**	0.003
PAX5α	–	–	−0.417**	0.000	−0.217*	0.035	−0.530**	0.000
PAX5β	−0.417	0.000**	–	–	0.138	0.180	0.510**	0.000
MMP2	−0.217*	0.035	0.138	0.180	0.001	–	0.180	0.080

Correlation is $^{*}p < 0.05$ is significant. $^{**}p < 0.01$ is highly significant.
[PAX5: paired box gene 5, MMP2: matrix metalloproteinase2, CA15-3: cancer antigen 15-3].

of *PAX5* in hepatocellular carcinoma resulted in suppressing growth and inducing apoptosis through upregulation of p53 and its downstream molecules, suggesting it as a novel tumor suppressor. Similarly, Proulx et al. [28] found that overexpression of *PAX5* induced apoptosis in multiple myeloma cells. On the other hand, Zhang et al. [29] revealed that *PAX5* interferes with p53 function through inhibition p53 gene transcription and hence protects cells from DNA damage-induced apoptosis in B lymphopoiesis. Regarding previous researchers that analyzed apoptotic activities by investigating *PAX5* isoforms, Vidal et al. [23] reported that the induce forced expression of *PAX5α*, resulted in a transcriptional up-regulation of p53 gene and reduced mRNA levels of the pro-survival genes BCL-XL and BCL-2 in MCF-7 cells with a consequently increased apoptotic cell death. However, the later study was carried out on breast cancer cell line contrary to the present work carried out on breast cancerous tissues.

Panth et al. [30] reported that MMP2 is an indicator of poor outcomes for oncologic patients as it plays a obvious role in cancer invasion and metastasis. We found that *PAX5α* expression was negatively correlated to MMP2. The down-regulation of *PAX5α* isoform accompanied by increase serum levels of MMP2 in our malignant patients highlight the importance of this interaction for tumor formation and metastasis. Similarly, Vidal et al. [21] found a significant negative correlation between *PAX5α* and MMP2 as recombinant *PAX5* overexpression reduced colony formation and promoted epithelial behavior in breast carcinoma cells.

The possibility that *PAX5* gene is aberrantly activated in tumors remains controversial. The oncogenic aggressiveness of *PAX5* in certain solid cancer types reported by some studies should be referred to differential expression of *PAX5* isoforms in specific types of malignant cells [7].

5. Conclusions

These findings indicated that *PAX5* might have a tumorgenetic role in breast cancer through down-regulation of *PAX5α* and the up-regulation of *PAX5β* isomers. The significant correlations of *PAX5* isomers with P53 and MMP2 could highlight their oncogenic properties and opens new prospects of therapeutic targets. However, the study should be performed on large number of the patients with investigating the molecular link between *PAX5α* and *PAX5β* as transcriptional factors to the expression of P53 and MMP2.

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