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Original Research

Potential role of PAX5 in Breast cancer: Relation to CD19

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ABSTRACT

Background: PAX5 is a transcriptional factor which is considered as one of the key molecules for regulation of many cancer associated genes. **Objectives:** To examine the status of PAX5 in human breast carcinoma and evaluate its role via CD19. **Patients and Methods:** PAX5 and CD19 expression in breast tissue samples were measured by RT-PCR in 90 female breast specimens including 50 cases of invasive ductal carcinoma, 25 cases with fibroadenoma and 15 non-pathological breast tissue as control group. **Results:** The best cut-off values for the investigated markers were determined by ROC curve. PAX5 at 0.05 had 92.0% sensitivity and 92.5% specificity while CD19 at 0.62 had 94.0% sensitivity and 95.0% specificity. Moreover, mean levels and positivity rates for PAX5 and CD19 showed significant difference among the three investigated groups ($p < 0.001$). PAX5 was higher in advanced grades ($p < 0.05$), stages ($p < 0.01$) and negative estrogen tumor receptors ($p < 0.05$). Additionally, PAX5 showed a significant positive correlation with CD19. **Conclusion:** PAX5 may be a potential biomarker having a possible role in breast cancer tumorgenesis through CD19.

KEY WORDS:Breast cancer; CD19; PAX5.

ABBREVIATIONS

AUC	Area under ROC curve
CD	Cluster of Differentiation
ER	Estrogen receptor
LN	Lymph node
PAX	Paired box
PAX5	Paired box 5
PR	Progesterone receptor
ROC	curve Receiver operating characteristic.
RT-PCR	Reverse transcription polymerase chain reaction

INTRODUCTION

Breast cancer incidence is rising worldwide with an increase in aggressive neoplasia in young women [1]. Although a number of significant advances have been made, the molecular mechanisms contributing to pathogenesis of breast cancer are poorly understood [2].

In considering that both normal developmental and neoplastic processes progress through common pathways as illustrated by the large number of developmentally regulated genes and signal transduction pathways with demonstrated roles in neoplastic progression [3]. The paired box (PAX) gene family is one such example, with family members implicated in both developmental and neoplastic processes [4]. PAX genes are a family of nine nuclear transcription factors that play a crucial and indispensable role in various developmental programs [5]. In humans, all the nine PAX genes are expressed during various stages of embryogenesis and development. In adults, most of the PAX genes are silent; however, they become selectively active during tissue repair and regeneration [6]. Interestingly, several of the PAX genes have been reported to be expressed in various cancers and are likely to contribute to the overall tumorigenesis [7].

PAX5 gene is located on chromosome 9q13 and was initially found to encode a nuclear DNA-binding protein in B cells [8] with the expected molecular weight of 50 kDa [9]. It influences B-cell differentiation and development by promoting lineage commitment while also inhibiting later differentiation events [10]. Its expression is switched off during the terminal differentiation of plasma cell [7]. Besides, PAX5 mRNA expression has also been reported in the developing central nervous system and adult mouse testis as well as in human adult brain tissue and some tissues of the male and female genital tracts [11].

Several studies have aimed to determine the downstream effectors of PAX5 by which it could regulate cell differentiation, proliferation, migration and survival that may contribute to oncogenesis [4]. Whereas increased PAX5 expression has been reported for a variety of malignancies, the precise role of PAX5 in cancer remains unclear, and conflicting evidence exists suggesting both tumor-promoting and tumor suppressor functions [12]. Although conflicting results are available regarding PAX5

signaling yet researches agree that the most important target for PAX5 is Cluster of Differentiation (CD) 19 [13, 14]. Studies revealed that PAX5 expression increases transcription of CD19 in B-acute myeloid leukemia (AML) cell lines [15] and in AML patients [16]. However, much remains to be elucidated regarding its precise role in breast cancer.

Therefore, the current study was conducted to investigate the possible role of PAX5 in the pathogenesis of breast cancer by correlating its expression to CD19 and different clinicopathological factors of breast cancer patients.

MATERIAL AND METHODS

Patients' database

A prospective analysis was performed between 2014 and 2015; it included 90 female Egyptian subjects, selected from General Surgery Department, Ain Shams University Hospitals, Egypt. The malignant group included 50 primary breast cancer patients (median age 45 years, ranging from 37 to 56 years), pathologically diagnosed as infiltrating ductal carcinoma. They were staged according to TNM classification of the American Joint Committee on cancer (AJCC) [17] and graded by the Nottingham grading system [18]. The benign group included 25 patients (median age 46 years, ranging from 34 to 58 years), pathologically negative for malignant hyperplasia, and were diagnosed as fibroadenoma. None of the patients were using oral contraceptives, hormones or vitamins. The control group included 15 normal volunteers' females who underwent plastic breast surgery (median age 42 years, ranging from 35 to 45 years). The pathological reports, estrogen and progesterone hormonal status were obtained from hospital records. This study has complied with the principles laid down in the Declaration of Helsinki, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, and recently amended at the 64th World Medical Assembly, Fortaleza, Brazil, October 2013. The entire protocol was approved by institutional ethical committee. All participants signed informed consent for participation in the study as required.

Samples collection and processing

Breast tissue samples were obtained from all women before any therapeutic measures. The samples were washed and transported in ice cold saline then kept frozen at -80°C until RNA extraction.

RNA extraction

Total RNA from the breast tissues was isolated with the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). The RNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm.

RT-PCR for PAX5 and CD19

Extracted RNA (2 μg) was reversed transcribed at 37°C for 60 minutes in a reaction mixture containing 100 pmol/l random hexamer (Pharmacia Biotech, Tokyo, Japan), 0.5 mmol/l each deoxynucleotide-5-triphosphate (dNTPs), 1X RT buffer (50 mmol/l Tris-HCl pH 8.8, 7.5 mmol/l KCl, 3 mmol/l MgCl2), 10 mmol/l dithiothreitol (DTT), 20 U of human placental ribonuclease inhibitor (Takara) and 20 U of RT (Superscript 11; GlBCO BRL, Gaithersburg, MD). The following primer sequences (0.6μM) were used for β-actin (forward: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and reverse: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3', GenBank: XM_006715764.1), PAX5 (forward: 5'-AATGACACCGTGCCTAGCGT-3' and reverse: 5'-GGTGGTGAAGATGTCTGAGT-3', GenBank: XM_005251481.3) and CD19 (forward: 5'-TAAGTCATTGCTGAGCCTAGA-3' and reverse: 5'-TCGCTGCTCGGGTTTCCATAA-3', GenBank: XM_006721103.2).

The cycling conditions for PAX5 and CD19 were initial denaturation step at 94°C for 10 minutes followed by 40 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 60°C and extension for 1 minute at 72°C followed by final extension step for 5 minutes at 72°C. While the cycling conditions for β-actin were initial denaturation step at 94°C for 10 minutes followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C and extension for 30 seconds at 72°C followed by final extension step for 5 minutes at 72°C. Electrophoresis was done through a 3% agarose gel containing ethidium bromide then the gel was visualized under UV illumination using gel documentation system (BioRad-Gel. 2000, Italy). The bands were analyzed by the "Quantity one" program (version 4.6.3, Bio-Rad, USA). Results were expressed as the density of PAX5 (fig. 1) and CD19 (fig.2) bands in comparison to the density of β-actin band for each sample.

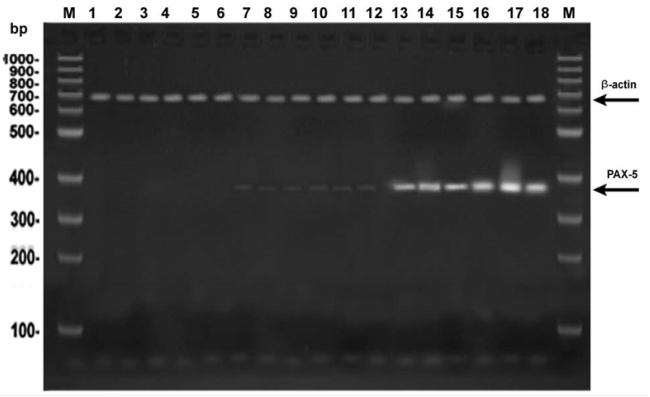

Fig. 1. Ethidium bromide-stained agarose gel electrophoresis showing RT-PCR products of PAX5 (381 bp) and β-actin (661 bp). DNA ladder (M), negative control (lane 1), healthy controls (lanes 2-6), benign breast patients (lanes 7-12) and malignant breast patients (lanes 13-18).

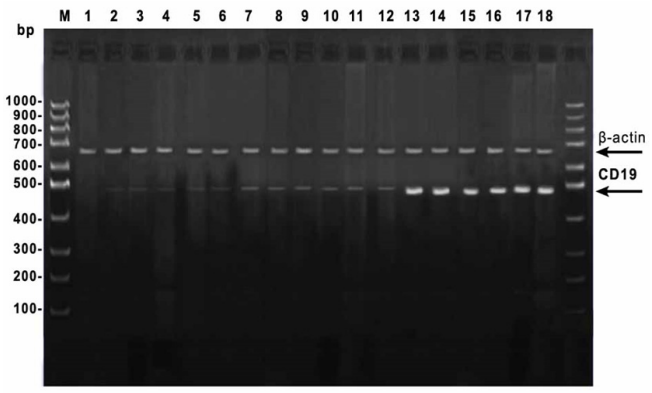

Fig. 2. Ethidium bromide-stained agarose gel electrophoresis showing RT-PCR products of CD19 (486 bp) and β-actin (661 bp). DNA ladder (M), negative control (lane 1), healthy controls (lanes 2-6), benign breast patients (lanes 7-12) and malignant breast patients (lanes 13-18).

Statistical analysis

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 19, SPSS Inc., Chicago, IL). Statistical comparisons were made using 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). Chi-square test was used to compare quantitative parameters between groups. Correlation between different variables was performed by Pearson`s correlation coefficient. The parameters were also correlated with the clinicopathological factors using the non-parametric test Kruskal-Wallis (χ^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 cut off points.

RESULTS

Breast tissues` RT-PCR products of ethidium bromide-stained agarose gel electrophoresis showed expression of PAX5 at 381 bp and CD19 at 486 bp, figure 1 and 2. Clinicopathological factors of the studied groups were shown in table (1). The expression levels of PAX5 and CD19 were significantly higher in malignant group compared to benign and control groups ($p<0.01$), with no significance difference between benign and control groups $p>0.05$, table 2. The overall diagnostic performance for the investigated markers was assessed by ROC curve by considering benign and healthy groups as the non-malignant group. The best cut off value for PAX5 was 0.05 with 92.0% sensitivity, 92.5% specificity and 0.93 Area under ROC curve (AUC) ,and for CD19 was 0.62 with 94.0% sensitivity, 95.0% specificity, 0.988 AUC, figure 3. The positivity rates of investigated parameters (above the calculated cut-off) were higher in malignant group compared to other groups ($p<0.01$), figure 4. Assessment of the relation between PAX5 and the different clinicopathological factors in our malignant patients revealed higher expression level of PAX5 in grade 3 ($p<0.05$), stage III ($p<0.01$) and in negative estrogen receptor tumor ($p<0.05$) than others, table 3. Additionally, PAX5 showed highly significant positive correlations with CD19, stage and grade ($p<0.01$), table 4.

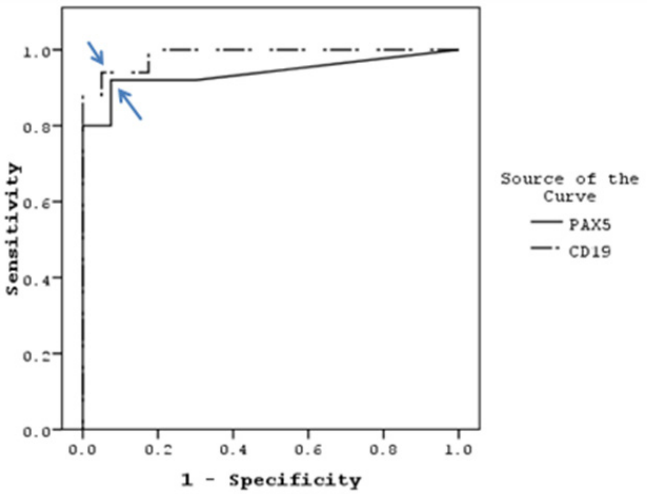

Fig. 3. Ethidium bromide-stained agarose gel electrophoresis showing RT-PCROC curve analysis for PAX5 and CD19 to calculate the best cut-off point to discriminate between malignant and non-malignant groups. Arrow denotes cut off point at 0.05 for PAX5 with 92.0% sensitivity, 92.5% specificity and 0.93 AUC, and 0.62 for CD19 with 94.0% sensitivity, 95.0% specificity and 0.988 AUC

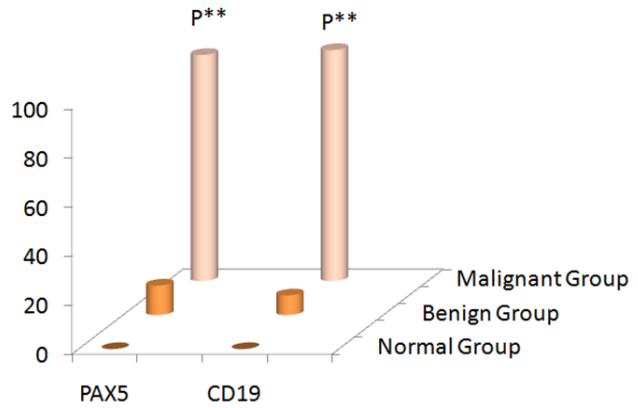

Fig. 4. Positivity rate of PAX5 and CD19 in malignant group compared with benign and control groups.

**p < 0.01 is highly significant between the three studied groups.

Table 1. Clinicopathological factors of the study groups

Clinicopathological factors	Normal No. (%)	Benign No. (%)	Malignant No. (%)	Statistics
Age (years)				
Median (range)	42 (35-45)	46 (34-58)	45 (37-56)	--------
Menopausal status;				
Premenopause	12 (80.0%)	13 (52%)	14 (28%)	χ^2: 4.28 p> 0.05
Postmenopause	3 (20.0%)	12 (48%)	36 (72%)	
Parity;				
0	5 (33.3%)	4 (16%)	4 (8%)	χ^2: 9.1 p> 0.05
1-2 children	6 (40%)	11 (44%)	16 (32%)	
≥3 children	4 (20.7%)	10 (40%)	30 (60%)	
Grade;				
Grade 1		----	2 (4%)	
Grade 2		----	36 (72%)	
Grade 3		----	12 (24%)	
Stages;				
Stage I		----	3 (6%)	
Stage II		----	29 (58%)	
Stage III		----	18 (36%)	
Tumor size:				
‹2cm			3 (6%)	
2-5cm			29 (58%)	
›5cm			18 (36%)	
+ve Estrogen R			35 (70%)	
+ve Progesterone R			40 (80%)	

p>0.05 is non-significant (NS).

Table 2. Expression levels of PAX5 and CD19 in the studied groups

Parameters	Normal Group	Benign Group	Malignant Group	p value
PAX5 Expression Level				
mean ±SD	0.005 ± 0.01	0.023 ± 0.06	2.27 ± 2.06	P1>0.05
Median	0.0	0.0	1.2	P2<0.01**
Range	0.0- 0.03	0.0- 0.3	0.0- 5.93	P3<0.01**
CD19 Expression Level				
mean ±SD	0.082 ± 0.02	0.34 ± 0.21	1.6 ± 0.6	P1>0.05
Median	0.0	0.33	1.47	P2<0.01**
Range	0.0- 0.09	0.0- 0.87	4.04- 2.84	P3<0.01**

p1= benign group versus healthy controls, p2= malignant group versus benign group and p3= malignant group versus healthy controls.

***p<0.01* is highly significant. *p>0.05* is non-significant.

DISSCUSION

PAX5 gene expression has been proposed to influence carcinogenic events in tissues of nonlymphoid origin by promoting cell growth and survival. However, in other cases, PAX5 products have opposing effects on proliferative activity [19] . Vouyovitch et al. [9] found that nuclear accumulation of PAX5, without increasing its expression level, in a JAK2-dependent manner is responsible for autocrine human growth factor induced proliferative disorders in human mammary carcinoma cells. However, Crapoulet et al. [20] reported that PAX5 has a negative effect on cancer cells migration and metastatic dissemination to distant sites through its inverse correlation with focal adhesion kinase 1 expression. In this study, PAX5 showed significant higher expression levels in malignant breast cancer compared to benign and control groups ($p<0.01$) with no statistical significant difference in the later two groups. By using ROC curve, PAX5 expression level at cut off value 0.05 had 92.0% sensitivity and 92.5% specificity with a high significant positivity rates in malignant group compared to other groups. Moreover, its expression levels were significantly high in advanced stage, grade and ER negative malignant tissues, which indicates its possible predictive role in tumorgenesis of breast cancer. Previous studies could explain the overexpression of PAX5 in malignant cases by activating its upstream signaling networks in cancer processes. Metastasis-associated protein-1 is a transcription factor that strongly correlates with invasiveness and angiogenesis in many cancers. This upstream factor could bind to enhancer sequence within the PAX5 gene in MCF-7 breast carcinoma cells and enhanced its expression [21].

Table 3. Relation between PAX5 expression and clinicopathological factors in the malignant group

Clinicopathological factors	PAX5 expression levels	
	Mean Rank	Positivity rate (No. of positive case/ No. of group)
Menopausal status		
Premenopause	24.56	13/14 (2.8%)
Postmenopause	27.93	33/36 (91.6%)
Parity		
0	18.13	3/4 (75.0%)
1-2 children	23.66	16/16 (100.0%)
≥3 children	27.47	27/30 (90.0%)
Grade		
Grade 1	26.75	3/3 (100.0%)
Grade 2	21.82	30/34 (88.2%)
Grade 3	36.33 χ^2: 8.96 *p: 0.011	13/13 (100.0%)
Stage		
Stage I	26.67	3/3 (100.0%)
Stage II	19.12	25/29 (86.2%)
Stage III	35.58 χ^2: 14.2 **p=0.001	18/18 (100.0%)
Estrogen Receptor		
Positive	24.18	32/36 (88.8%)
Negative	34.86 χ^2: 4.9 *p=0.026	13/14 (92.8%)
Progesterone Receptor		
Positive	25.3	37/41 (90.2%)
Negative	35.33	9/9 (100.0%)

*p <0.05 is significant. **p <0.01 is highly significant.

Table 4. Pearson Correlation (r) between the investigated parameters

Parameters	PAX5		CD19	
	r	p	r	p
Age	0.028	0.79	0.05	0.58
PAX5	-----	-----	0.32**	0.002
CD19	0.32**	0.002	-----	-----
Stage	0.448**	0.001	0.20	0.14
Grade	0.414**	0.003	0.19	0.18

Correlation is **highly significant at *p<0.01* level.

CD19 protein is expressed on the surface of all B-lymphoid cells with the exception of terminally differentiated plasma cells and has been implicated in the control of proliferation and differentiation. Its promotor contains a high binding affinity to PAX5 transcriptional factor that regulates its transcription [22]. In the current study CD19 expression levels and positivity rates were significantly high in the malignant group compared to other groups with 94.0% sensitivity and 95.0% specificity. Moreover, CD19 showed a significant positive correlation with PAX5. Similarly, Walter et al. [23] reported a positive correlation between PAX5 and CD19 expression in AML cells. Moreover, they demonstrated that PAX5 binds to the promoter and enhancer of CD19 gene causing remodeling of the chromatin structure at the promoter suggesting functional consequences for the expression of PAX5 in leukemic cells. Furthermore, Danbara et al. [24] reported that silencing of the PAX5β gene was associated directly with loss of CD19 gene expression in lung and breast cancer cells. Researchers concluded that the exact role of CD19 in breast cancer tissues was unclear and wondered whether its higher level was due to mammillary cells overexpression or due to lymphocytic infiltration in the solid tumors. Indeed, they stated that the regulation of CD19 expression by PAX5 might modulate immune response with selective growth advantage of breast cancer. Additionally, they recommended further analysis by immunohistochemistry to illustrate the neoplastic cells which express B-cell markers [25].

CONCLUSION

All these findings indicated that PAX5 is a potential biomarker that discriminates between malignant and non-malignant breast tissues with higher sensitivity and specificity. Its significant relation to CD19 may support the role of CD19 as a PAX5 downstream effector in breast cancer tissues. Further studies are necessary to understand the precise role of the different PAX5 isoforms in the molecular pathogenesis of breast cancer.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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