ER, p53 and MIB-1 are Significantly Associated with Malignant Phyllodes Tumor

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Background: Fibroadenomas (FA) are common while phyllodes tumors (PT) are rare and both tumors are composed of epithelial and stromal components. We evaluated the expression status of ER, Bcl2, p53, and MIB-1 protein in these tumors.

Methods: One hundred and ninety-three tumors comprising of 117 FAs and 76 PTs were examined using immunohistochemistry on tissue microarray.

Results: The mean age of patients with FA was 28.5 years while the mean ages of patients with benign, borderline and malignant PTs were 41.7, 48.6 and 42.1 years, respectively. Also all types of PTs were large (>5cm). ER showed a strong nuclear staining in the epithelial component of all tumors while ERβ immunoreactivity was detected in both the epithelial and stromal components of FA and PT. ERβ (p<0.001), and p53 (p=0.006) in the stromal component were associated with tumor size. p53 expression was significantly associated with both the epithelial and stromal components of malignant PTs (p<0.05). In the PT, the decreased expressions of p53 and MIB-1 were significantly different with positive Bcl2 protein expression in the epithelial component (p=0.000). In addition, MIB-1 was also found to be associated with ER and ERβ in the stromal component (p=0.000).

Conclusions: The expression of p53 with tumor size and histological grade in PT may increase the risk for malignancy.

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Key words: estrogen receptor, fibroadenoma, immunohistochemistry, MIB-1 protein, phyllodes tumor, p53 antigen

At a Glance Commentary

Scientific background of the subject

Phyllodes tumors (PT) are rare. PTs may be classified as benign, borderline and malignant subtypes. The PT has to be differentiated from the fibroadenomas because PT can recur. We assessed the usefulness of these markers in distinguishing benign from malignant tumors by tissue microarray.

What this study adds to the field

ER, p53 and MIB-1 expressions are significantly associated with the pathogenesis of PTs. MIB-1 expression was associated with ERα and ERβ in the stromal component. The expression of p53 with tumor size and histological grade in PTs may increase risk for malignancy.

Fibroepithelial tumors of the breast illustrated as a proliferation of epithelia and stroma component. This tumor group ranges from benign to malignant; includes fibroadenomas and phyllodes tumors (PTs).[1] Fibroadenomas (FAs) are the most common fibroepithelial tumor among young women, but it can be also diagnosed in postmenopausal women. It is composed of both glandular and connective tissue, characterized by hyperplasia and abnormal lobular breast units.[2-4] The PTs are rare neoplasms and accounting for less than 1% of all primary breast tumors.[5-7] PTs show predominantly of FA criteria, with a leaf-like projection cells and more cellular connective tissue stroma component.

PTs may be classified as benign, borderline and malignant subtypes according to gross and microscopic features including margin appearance, cellular pleomorphism, stromal cellularity, mitotic activity and

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stromal distribution.[1,6-8] Apoptosis is one of the factors that play a key role in the development and growth regulation of normal and neoplastic mammary tissues.[9,10] Bcl2 is an oncoprotein that plays a role in apoptosis; by blocking programmed cell death, discourages the promotion of cellular proliferation and induces tumor development.[10,11]

The expression of Bcl2 is associated with cells that are protected from apoptosis such as stem cells or epithelia that undergo hyperplasia; including breast and prostate and its expression is related to steroid hormone receptor.[10,12]

The estrogen receptor (ER), is ligand-activated transcription factors that normally present in normal and benign lesions of the breast.[10,13] Generally, ER has been implicated in both normal and neoplastic mammary tissue as a DNA binding transcription factor and induces cell proliferation.[4,14] It was first cloned as ER located on the long arm of chromosome 6q25 and is now known as ERα. The second type of ER is ERβ, located on chromosome 14q22-24.[13] ERα was reported to express mainly by epithelial cells in fibroepithelial tumors, while was commonly difficult to define the presence of ERα in the stroma component.[15]

Ki-67 antigen is a cell proliferation-related protein that can be detected with monoclonal antibody MIB-1 and to evaluate proliferative activity in different types of tumor.[16,17] Ki-67 is linked to cell cycle specially expresses during late G1, S, M and G2 phase of the cell cycle, is undetected in cells of the G0 phase.[6,18] It is observed as a marker of cell proliferation and the best indicator in determining the growth fraction of cells.[6]

Expression of the proliferation marker Ki-67 has been reported to vary among histological categories of PT as well as between PTs and FAs and was also reported in several studies to show a correlation between MIB-1 positivity and the histological grade.[7,19,20]

Besides, p53 immunohistochemical expression, commonly used as an identification for tumor-suppressor gene mutation, has been also correlated with tumor grade.[7,21] It is located on the short arm of chromosome 17p13, is a well-known tumor suppressor gene, plays a role in the regulation of normal cell growth and division, DNA repair and apoptosis.[22]

The main aim of the present study was to determine the expression status of ERα, ERβ, Bcl2, p53, and MIB-1 protein in fibroepithelial lesion by tissue microarray. We determined the relationship between the immunostaining expression with clinicopathological criteria such as age, tumor size and tumor types and to assess the usefulness of these markers in distinguishing benign from malignant tumors.

METHODS

Patients

Prior ethical approval was taken from Institutional ethics committee to conduct the study. The study population consisted of 193 patients and all samples with patient's data were collected from Department of Pathology, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). A total of 117 FAs and 76 PTs (45 benign PT, 17 borderline PT and 14 malignant PT) histology slides were examined through microscope and evaluation by pathologists to determine the spot area; consisting of both epithelia and stroma components of fibroepithelial lesions. The PTs were graded into benign, borderline and malignant tumor, according to WHO classification.[23]

Tissue microarray

Tissue microarray (TMA) was conducted to all selected cases using 0.6-mm diameter punch kit MTA Booster (Alphelys, France). Approximately 50-70 cases were punched into a single TMA block and quadruplicate, giving a total of 16 TMA blocks.[24]

Immunohistochemistry (IHC)

Immunohistochemical staining was performed manually to determine the expression of selected biomarkers in epithelia and stroma using DAB horseradish chromogen. Three micrometer sections were cut onto poly-L-lysine-coated slides and baked at 60ºC for 30 minutes. Deparaffinization in xylene and rehydration through graded alcohol followed by treating with antigen retrieval for 40 minutes at 98ºC. The sections then were cooled down in room temperature and incubated with blockage agent, hydrogen peroxide for 10 minutes followed by primary antibody incubation for 30-60 minutes in room temperature. The primary antibody and their dilution were shown in Table 1.

Incubation with Dako EnVision was followed by DAB chromogen detection and then counterstained in hematoxylin solution. Sections of tonsil, colon adenocarcinoma, breast carcinoma and endometrium shown strong staining (3+) were used as a positive control for Ki-67 (clone MIB-1) and Bcl2, p53, ERα and ERβ, respectively. Negative controls were performed by omitting the primary antibody. IHC staining of p53 and Ki67 were interpreted as positive when more than 10% staining of the tumor nuclei were detected.[25] The ER and Bcl2 were required as positive reactivity when 10%

Table 1: Clone and optimal dilution for each of the primary antibodies

Primary antibody	Clone	Optimal dilution (min)
bcl2	124, DAKO	1:50 (30)
ERα	1D5, DAKO	1: 75 (30)
ERβ	MCA1974S, Serotec	1:10 (30)
Ki-67	MIB-1, DAKO	1:75 (60)
p53	DO7, DAKO	1:150 (30)

of nuclear or cytoplasm staining of at least 2+ intensity of expression detected.[26] Several cases were missing in immunohistochemical analysis due to loss of TMA spot.

Statistical analysis

The Chi-square test was performed to evaluate the association of biomarkers expression with tumor size and tumor types, while the co-expression between two biomarkers was evaluated by the McNemar's test. Differences were considered to be statistically significant at $p < 0.05$. All analyses were carried out using the software Statistical Packages for the Social Sciences, SPSS v12.0 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

An increasing trend of tumor size and age was detected for benign and borderline tumors with the exception of malignant PT. The mean age of patients with FA was 28.5 years (range, 10-59). The mean age for benign, borderline and malignant PTs was 41.7 (range, 19-74), 48.6 (30-78), and 42.1 (25-67) years, respectively. The mean tumor sizes were 3.4 ± 2 cm, 6.5 ± 4.4, 12.6 ± 8.0 and 11.0 ± 8.4 cm for FA, benign PTs, borderline PTs and malignant PTs, respectively. Overall, 61 out of 193 cases had tumor size less than 3 cm, 73 cases range between 3 and 5cm and 59 cases were more than 5 cm. Most of the FA cases had small size tumor which 45.3% (53/117) cases were less than 3 cm. Meanwhile, all type of PT were bigger in size whereas 48.9% (22/45), 82.4% (14/17) and 64.3% (9/14) cases of benign, borderline and malignant PT, respectively, had tumor size more than 5 cm [Table 2].

Figure 1 showed the distribution of biomarkers in stromal component versus tumor size between four cases groups. ERα positive was expressed only in a case (33.3%) of malignant PT with tumor size ranges 3-5 cm [Figure 1(A)]. Five of 11 (45.5%) cases of FA with the tumor size of >5 cm were ERβ-positive, higher than other group of tumor size in FA [Figure 1(B)]. The expression also showed an increased pattern of ERβ with tumor size for all group except borderline PT. Compared to ERβ, malignant PT showed loss of expression of Bcl2 as the tumor size increased [Figure 1(C)]. Immunoreactive p53 was seen in the borderline and malignant PTs [Figure 1(D)] while MIB-1 was mainly expressed in the larger size tumor of PT, >5cm [Figure 1(E)]. No statistically significance was found between biomarkers expression in stromal component with tumor size in every single group. But, when we analyzed all cases together, the expression of ERβ and p53 showed significant association with bigger tumor size ($p < 0.001$, $p = 0.006$).

Expression of study markers in FA and PTs (benign, borderline and malignant) by tissue microarray were shown in Figure 2: FA [Figure 2(A)], benign PT [Figure 2(B)], borderline PT [Figure 2(C)], and malignant PT [Figure 2(D)]. ERα showed a strong nuclear staining in the epithelial component of all tumors, while ERβ immunoreactivity was detected in both epithelial and stromal components of FA and PTs. Only the stroma of the malignant PTs was shown [Figure 2(D)]. Bcl2 staining was detected in the cytoplasm of epithelial cells and also expressed in stromal component of FA and PT. p53 was strongly expressed in the stroma of borderline and malignant PT, respectively, The results of

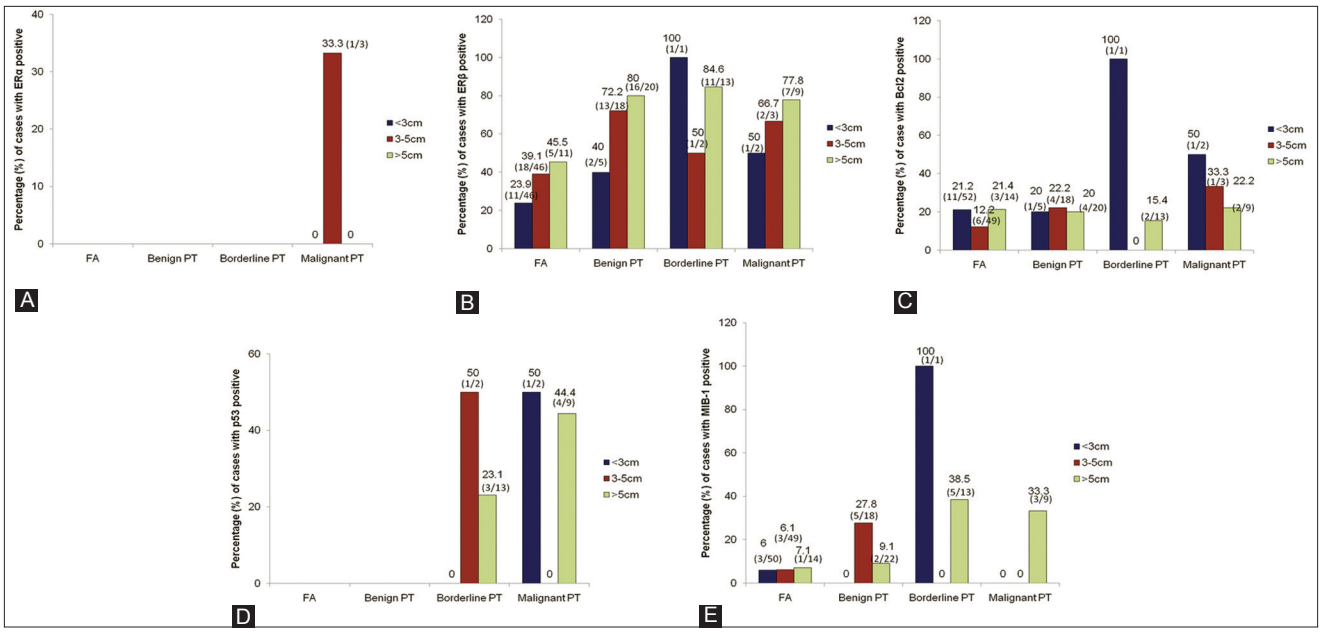

Figure 1(A-E): Distribution of ER , ERβ, Bcl-2, p53 and MIB-1 positivity in stromal component of fibroadenoma and phyllodes tumors with tumor size (%) (number of cases in parenthesis).

Table 2: Tumor size distribution between fibroadenoma and phyllodes tumors

	FA	Benign PT	Borderline PT	Malignant PT	Total
n	117	45	17	14	193
Age (years)					
Mean±SD	28.5±11.8	41.7±12.9	48.6±10.4	42.1±12.3	
(Range)	(10-59)	(19-74)	(30-78)	(25-67)	
Size tumor (cm)					
Mean±SD	3.4±2.0	6.5±4.4	12.6±8.0	11.0±8.4	
(Range)	(0.4-11.5)	(2.3-23.0)	(1.5-32.0)	(2.5-27.0)	
<3 cm	53 (45.3%)	5 (11.1%)	1 (5.9%)	2 (14.3%)	61
3-5 cm	50 (42.7%)	18 (40.0%)	2 (11.8%)	3 (21.4%)	73
>5 cm	14 (12.0%)	22 (48.9%)	14 (82.4%)	9 (64.3%)	59

Abbreviations: FA: Fibroadenoma; PT: Phyllodes tumor

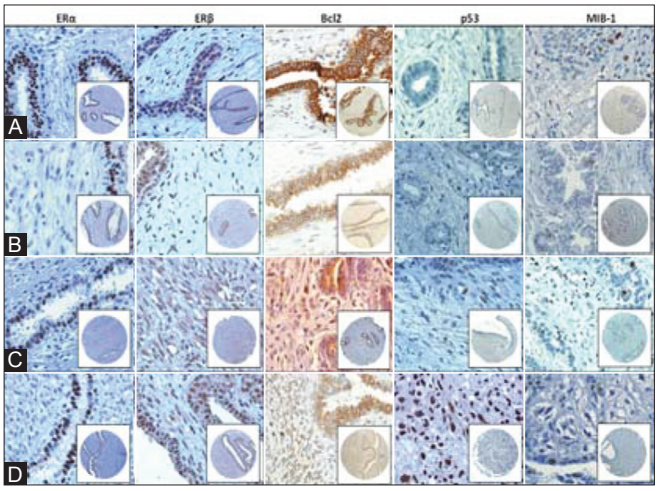

Figure 2: An immunohistochemical staining for ER , ERβ, Bcl2, p53 and MIB-1 (in rows), performed on tissue microarray sections of (A) fibroadenoma, (B) benign phyllodes tumor, (C) borderline phyllodes tumor, and (D) malignant phyllodes tumor as shown in the column. [original magnification x200 (insert, x40)]

overexpression of biomarkers are listed in Table 3. Both types of ER and Bcl2 expression were greater in epithelia of benign than malignant tumors. However, the rate of ERβ expression was higher in the stromal component than ERα, and that ERα appeared undetected in FA, benign and borderline PTs. However, ERβ showed an excellent pattern in epithelial component with high expression in benign cases and reduced with malignancy; 84.3% of, 76.7%, 76.5% and 50% in FA, benign PT, borderline PT and malignant PT; respectively, but was not statistically significant. ERα was mainly expressed by epithelial cells and only 7.1% (1/14) was significantly expressed in the stroma of malignant PT while absent in borderline and benign PTs ($p = 0.006$). The present study also showed a greater percentage of expression of p53 and MIB-1 in borderline and malignant tumors. p53 expression was significantly associated with grade, in both epithelial and stromal ($p < 0.05$) components. The p53 was absent in FA while expressed in 2.3% (1/44) cases of benign PT for epithelial component. Immunostaining for p53 was found to be increased with histological grade; 5.9% (1/17) and 20% (1/5) cases of borderline and malignant PT, respectively, for epithelial component. Stromal component was absent in both benign tumors (FA and benign PT), and showed greater expression for 23.5% (4/17) of borderline and 35.7% (5/14) of malignant PT ($p = 0.000$). Meanwhile, MIB-1 expression in stromal component was also significantly increased in borderline and malignant tumors when compared to FA and benign PT ($p = 0.003$). About 35.3% (6/17) and 21.4% (3/14) cases of borderline and malignant PT, respectively, were positive for MIB-1 in stromal component; higher than 15.6% (7/45) cases of benign PT [Table 3].

Overall, significant differences between the expression of Bcl2 with p53 and MIB-1 were observed in the epithelial component of PTs [Table 4A]. Forty-two of 61 (68.85%) cases were correctly detected by Bcl2, whereas only two (3.28%) were positive for p53 ($p = 0.000$). Decreased MIB-1 counts were also significantly associated with positive Bcl2 protein expression and vice versa in 26 (43.3%) and four (6.7%) of 60 cases, respectively ($p = 0.000$). Our data also indicated that the expression of MIB-1 was statistically different with ERα in stromal component ($p = 0.000$; Table 4B). Similarly the coexpression between MIB-1 and ERβ in stromal component was also significantly different when 16 of 74 (21.62%) cases showed MIB-1positivity, whereas 54 (72.97%) cases were ERβ positive ($p = 0.000$ by McNemar test).

DISCUSSION

Tissue development is the result of a balance between cell proliferation, differentiation and apoptosis.[10] Proliferation of fibroepithelial tumor is mainly in the stromal component, followed by proliferation of epithelia cells.[15] The stromal elements were considered as the neoplastic component and therefore believed as the determinant of biological activity.[27]

The known Bcl2 protein is usually expressed in normal tissue and benign proliferative lesions, as they discourage the

Table 3: Biomarker expression in epithelial and stromal components of fibroadenoma and phyllodes tumors

		FA *n*=117 (%)	Benign PT *n*=45 (%)	Borderline PT *n*=17 (%)	Malignant PT *n*=14 (%)	*p*-value
ER	Epithelia	73/114 (64.0%)	25/40 (62.5%)	10/16 (62.5%)	5/9 (55.6%)	0.965
	Missing	3	5	1	5	
	Stroma	0	0	0	1/14 (7.1%)	0.006*
ERβ	Epithelia	86/102 (84.3%)	33/43 (76.7%)	13/17 (76.5%)	3/6 (50%)	0.169
	Missing	15	2	0	8	
	Stroma	34/103 (33.0%)	31/43 (72.1%)	13/17 (76.5%)	10/14 (71.4%)	0.000*
	Missing	14	2	0	0	
Bcl2	Epithelia	96/115 (83.5%)	24/39 (61.5%)	14/17 (82.4%)	4/10 (40%)	0.001*
	Missing	2	6	0	4	
	Stroma	20/115 (17.4%)	9/43 (20.9%)	3/17 (17.6%)	4/14 (28.6%)	0.765
	Missing	2	2	0	0	
p53	Epithelia	0	1/44 (2.3%)	1/17 (5.9%)	1/5 (20%)	0.003*
	Missing	0	1	0	9	
	Stroma	0	0	4/17 (23.5%)	5/14 (35.7%)	0.000*
MIB-1	Epithelia	37/113 (32.7%)	9/44 (20.5%)	9/17 (52.9%)	2/5 (40%)	0.097
	Missing	4	1	0	9	
	Stroma	7/113 (6.2%)	7/45 (15.6%)	6/17 (35.3%)	3/14 (21.4%)	0.003*
	Missing	4	0	0	0	

Abbreviations: FA: Fibroadenoma; PT: Phyllodes tumor; *:$p<0.05$ showed significant analysis

promotion of cell proliferation by inhibit apoptosis.[10] There was a significant difference of the Bcl2 expression between the benign (61.5%), borderline (82.4%) and malignant PT (40%) in epithelia, but was not statistically significant in the stroma. Cells overexpressing Bcl2 gene product have been shown to cause a rapid arrest in the G1 phase of the cell cycle[28] suggesting a role of Bcl2 in the transition from G0/G1 to S phase.[29] MIB-1 is expressed throughout the cell cycle in proliferating cells; however, not in cells in either the G0 phase or in the early phase of G1. In view of the lack of MIB-1 expression in Bcl2-positive tumor cells, it is possible that the PT analysed in this experiment are represented by cells in either the G0 or the early first gap phase. Our findings also suggest the role of Bcl2 in regulating cell cycle and proliferation of PTs.

Bcl2 expression can be detected in many hormonally regulated cells, such as epithelia cells of normal breast and ER/PR-positive breast carcinomas.[10,30,31] Furthermore, Bcl2 has been shown to be an important downstream mediator of estrogen action survival of cancer cells.[10] However, in our study, Bcl2 was not found to be significant associated with the expression of steroid hormone receptors such as ER, compared to other studies.[4,10]

Estrogen has a significant role in promoting malignancy in breast and potentially to be a mediator of the mammary gland since it is necessary for development and metastasis.[13] The results of the present study showed a significant increasing trend of ER expression with malignancy in the stromal component ($p < 0.05$). In other studies, ER was regularly present in normal and benign lesions of breast and metastasis tumors were usually negative for ER.[10] Similarly in our study, ERα expression was more common in benign tumor, but lower expression was detected in epithelial cells of malignant PT. On the other hand, ERβ showed an excellent pattern in epithelial component with high expression in benign cases and reduced with malignancy but was not statistically significant.

A study reported that ERα was mainly expressed by epithelia cells while no expression in the stromal cells[15] compared to the present study, only 7.1% ERα expression in malignant PT and absent in borderline and benign PT. Previous studies demonstrated that ERβ was found in stromal cells in the FA and PT.[13,15] We opine that ERβ showed better expression than ERα which was highly detectable in the stromal component. The greater expression of ERβ suggests that it may be the main ER in the breast. So that, Speirs and colleagues believed that cells initially considered ERα negative may actually be expressing ERβ.[13]

Recent study demonstrated the expression of ER was related to the proliferative index of the tumors measured by immunolabelling with antibodies against Ki-67 and indicating a good prognosis with endocrine therapy.[10] The results of the present study also showed significant association between MIB-1 expression with ERα and ERβ in stromal component. Prodifferentiative function on ERβ on stromal cells was suggestive to be associated with

Table 4: Association between biomarkers in (A) epithelial and (B) stromal components of phyllodes tumor

(A)

	Total	Bcl2 + No. cases	Bcl2 - No. cases	*p*
ERα	61			
+		31	7	0.804
-		9	14	
ERβ	61			
+		33	12	0.359
-		7	9	
p53	61			
+		2	0	0.000
-		40	19	
MIB-1	60			
+		14	4	0.000
-		26	16	

Abbreviation: No:number

(B)

	Total	MIB-1 + No. cases	MIB-1 – No. cases	*p*
ERα	73			
+		0	1	0.000
-		16	56	
ERβ	74			
+		15	39	0.000
-		1	19	
Bcl2	74			
+		6	10	1.000
-		10	48	
p53	75			
+		4	5	0.143
-		12	54	

Abbreviation: No.: number

expression of ERβ.[15] Another study suggested that cells that were immunopositive for ERα very rarely undergo proliferation, as determined by the lack of expression of the cell cycle associated antigen Ki-67 and proliferative cell nuclear antigen[13] which was similar to the present study. Expression of the proliferation marker, Ki-67 has been reported to be different between histological categories.[7,21]

Previous studies and our study have shown a correlation between MIB-1 positivity and the histological grades.[7,19,32] MIB-1 negativity or low expression indicated a very low proliferation rate, apparently noticeable in FA and benign PT. MIB-1 positivity in more than 10% of cells within a benign PT has been used as a risk for malignant change.[17,33] Our results showed a significant difference of MIB-1 positivity in stromal components. The expression of MIB-1 in our study in stroma was 15.6%, 35.3% and 21.4% in benign, borderline and malignant PTs, respectively, within the range of an earlier report by Tse *et al.*[32]

Published studies showed that Ki-67 and p53 expressions correlated well with the morphological grading of PT.[7,17,19] p53 is useful as independent criteria for evaluating the malignancy of PT and its expression tends to be greater in the PT with a higher malignant potential.[34] In the present study, low levels of expression were significantly found in the epithelium, 2.3% (1/44), 5.9% (1/17), 20% (1/5) of benign PT, borderline PT and malignant PTs, respectively. While in the stromal component there were high levels of expression which was significantly associated with p53 and histological grade with 23.5% (4/17) and 35.7% (5/14) of borderline PT and malignant PTs, respectively. Greater expression in the stroma component supports the assertion that the stroma rather than the epithelia component is neoplastic.[27] Even so, interactions between both stroma and epithelia, may influence the pathogenesis of PTs.[27] In addition, the negativity of p53 in the stromal cells confirmed the benign of the PT.[21,32,35] In this study, none of FA and benign PTs expressed p53 in the stroma.

Neither MIB-1 nor p53 expression may distinguish benign from malignant PT in diagnostically difficult cases such as borderline or low proliferative malignant cases. Ki-67 antigen presents as the true proliferation activity and overexpression of p53 is caused by progression of cells from benign to malignant tumor.[17] The association between MIB-1 and p53 expression with histological grade, indicates that their immunoreactivity in benign breast lesion may increase risk for malignancies and demonstrate to be possible indicator in PTs while ER expression status plays the role in determining the treatment in breast tumor.[36] p53 expression in PT has been found to be associated with histological features of malignancy, and the positivity increases in those tumors which progress from benign to malignant phenotypes.[6]

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