RESEARCH ARTICLE

Expression of DNA Methylation Marker of Paired-Like Homeodomain Transcription Factor 2 and Growth Receptors in Invasive Ductal Carcinoma of the Breast

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Abstract

Background: Paired-like homeodomain transcription factor 2 (PITX2) is another new marker in breast carcinoma since hypermethylation at P2 promoter of this gene was noted to be associated with poor prognosis. We investigated the expression of PITX2 protein using immunohistochemistry in invasive ductal carcinoma and its association with the established growth receptors such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth receptor 2 (HER2). Methods: We conducted a cross sectional study using 100 samples of archived formalin-fixed paraffin embedded tissue blocks of invasive ductal carcinoma and stained them with immunohistochemistry for PITX2, ER, PR and HER2. All HER2 with scoring of 2+ were confirmed with chromogenic in-situ hybridization (CISH). Results: PITX2 protein was expressed in 53% of invasive ductal carcinoma and lack of PITX2 expression in 47%. Univariate analysis revealed a significant association between PITX2 expression with PR (p=0.001), ER (p=0.006), gland formation (p=0.044) and marginal association with molecular subtypes of breast carcinoma (p=0.051). Combined ER and PR expression with PITX2 was also significantly associated (p=0.003) especially in double positive cases. Multivariate analysis showed the most significant association between PITX2 and PR (RR 4.105, 95% CI 1.765-9.547, p=0.001). Conclusion: PITX2 is another potential prognostic marker in breast carcinoma adding significant information to established prognostic factors of ER and PR. The expression of PITX2 together with PR may carry a very good prognosis.

Keywords: PITX2 - hypermethylation - IHC - invasive ductal carcinoma - growth receptors - prognostic marker

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Introduction

Breast carcinoma is the most common malignancy in women. The National Cancer Registry of Malaysia estimates approximately 1 in 20 women in this country develop breast cancer in their lifetime with a rate differs among the three main races. The commonest age at presentation is between 40-49 years, with just over 50% of the cases under the age of 50 years, 16.8% below 40, and 2% under 30 (Yip et al., 2006).

In the last few years, the role of DNA methylation in carcinogenesis has been a topic of considerable interest. Alteration in DNA methylation is the most common epigenetic modification which occurs in variety of tumour including breast cancer (Nimrich et al., 2008). Methylation of DNA occurs when there is covalent addition of methyl groups to the genomic DNA without changing the DNA sequence. Many recent studies revealed that epigenetic changes are more prominent than genetic changes in most cancers.

In breast cancer, there are a few genes that commonly hypermethylated such as $P16^{NK4A}$, Estrogen Receptor (ER) α, Progesterone Receptor (PR), BRCA1, GSTP1,TIMP-3 and E-cadherin (Das and Singal, 2004). However, for the screening purposes, no suitable gene or set of genes consistently hypermethylated in breast cancer but not in healthy tissue or detectable in serum or plasma have yet been found. Recently, a paired-like homeodomain transcription factor 2 (PITX2) DNA methylation has been validated in early breast cancer in a large, independent, multicenter cohort using a validated assay and it was found to be a potential biomarker for outcome prediction in breast cancer (Maier et al., 2007; Nimrich et al., 2008; Harbeck et al., 2008; Esteller, 2008).

PITX2 is one of DNA methylation marker and it acts as a transcription factor. It plays an important role in development and maintenance of anterior structures such as the eye, tooth and abdominal organs. It also acts as a transcriptional regulator involved in basal and hormone-regulated activity of prolactin. Mutation in PITX2 genes are the cause of Rieger syndrome type 1 (RIEG1), iridogoniodysgenesis syndrome 2 (IGDS2) and

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Peters anomaly. The role of PITX2 DNA-methylation in cancer is unknown but previous study showed methylated PITX2 at P2 promoter was significantly associated with poor outcome.

Maier et al (2007) have done a study to identify and validate DNA methylation of PITX2 marker associated with very good outcome in node negative and ER/PR positive breast cancer using a methylation microarray. They concluded that DNA methylation of PITX2 showed the strongest correlation with distant recurrence in node negative, hormonal receptors positive, tamoxifen treated breast cancer (Maier et al., 2007). Harbeck et al (2008) continued a study to establish determination in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue using a real-time polymerase chain reaction (PCR) method as a marker for outcome prediction in tamoxifen treated, node negative breast cancer patient. The result show that PITX2 methylation added significant information to establish prognostic factors such as tumour size, grade and patient age (Harbeck et al., 2008).

Widschwendter et al (2008) have conducted an epigenotyping study in peripheral blood cell DNA and proved that DNA methylation could be modulated by environment (like estrogen) and indicate the predisposition to develop a cancer (Widschwendter et al., 2008). However, whether DNA methylation profile will be able to serve as a new tool to predict the risk of breast cancer is uncertained.

Review of literatures make the researcher believe that there is a role of PITX2 methylation marker in pathogenesis and prognosis of breast cancer. But till date, there is no study done solely on the relation of PITX2 marker by using immunohistochemistry (IHC) with invasive breast cancer in FFPE tissue. Since the role of ER, PR and human epidermal growth factor receptor 2 (HER2) as the prime prognostic indicators had been long time established, the relationship of PITX2 methylation marker with these receptors should be determined. However no study has been done so far to determine the relationship of PITX2 with these receptors. Therefore this research is needed in order to verify the specific role of PITX2 methylation in invasive breast cancer, thus demonstrating a new prognostic marker as well as a guide for the response of hormonal therapy.

Materials and Methods

Study design and samples

A cross sectional study was conducted. 100 samples of retrieved paraffinized archival tissue blocks from mastectomy, lumpectomy and wide excision specimens with final histopathologic diagnosis of invasive ductal carcinoma of no special type were selected. The demographic data and histopathological examination reports were obtained from registry book at the Department of Pathology, Hospital University Science Malaysia, Kota Bharu, Kelantan. The histopathological reports were studied to obtain information regarding clinicopathological parameters such as tumour size, lymph node involvement, lymphovascular permeation and tumour grade. The specimen had been reported by various independent pathologists in the Pathology Department of Hospital University Science Malaysia.

Immunohistochemistry

Indirect immunohistochemical staining was performed on 3μ thickness paraffin sections for ER, PR, HER-2 and PITX2. PITX2 antibody (ab32832) is a rabbit polyclonal antibody, isotype IgG and protein A purified. It is a synthetic peptide derived from a region between amino acids 20-69 of Human PITX2 (Splice variant C. SwissProt ID Q99607-2). 1:100 dilution (10μl antibody +990μl antibody diluent) was applied to the sections and incubated overnight at 40^{o}C using immunostainer squenza. ER antibody (clone number; TE111.5D11), Abcam, ab16460 is a mouse monoclonal to estrogen receptor with IgG1 isotype and IgG fraction purified. PR antibody (clone number; Y85), ab32085 is a Rabbit monoclonal to progesterone receptor. The isotype is IgG and the purity is IgG fraction. Both were applied to the sections at 1:50 dilution and incubated. HER2 antibody (Dako, A 0485) also synonyms for antigen of HER2/neu, ErbB2 and p185^{HER2} is a Rabbit polyclonal to HER-2. It was applied to the sections at dilution of 1:400 and incubated for 30 minutes at room temperature. Buffer solution used for heat induced-epitope retrieval (HIER) in all PITX2, ER, PR and HER2 was Tris-EDTA buffer PH9.0 from Dako. The antigen retrieval was done using a pressure cooker method (DAKO, Denmark). 2 drops HRP Polymer applied on each section and incubated for 1 hour at room temperature. Di-amino benzidine (DAB) from DAKO, a chromogen was applied to the sections and incubated for 1 minute. Counter-staining was performed with a 3 or 4 dip in Hematoxylin.

Evaluation of immunoreactivity

PITX2 interpretation

Semiquantitative assessment was done under light microscopy. At the scanning view (magnification of 40X), an area of high population of tumour cells with the staining showing at least similar intensity with control slide were selected. Under high power field (magnification of 400X), a count of 100 tumour cells were made. The number of cells showing cytoplasmic staining were counted into percentage. The cytoplasmic staining was classified into mild and strong intensity. When the staining intensity was lower than the control intensity, it was considered mild cytoplasmic intensity, but then if the intensity was similar or stronger than the control intensity, it was classified as strong cytoplasmic intensity. Then, a histogram was plotted based on the percentage of cytoplasmic staining (Figure 1). Cut-off point of positivity was determined based on staining distribution and intensity of the cytoplasmic staining.

ER and PR interpretation

Immunostained slides were scored using Allred scoring method. Firstly, a proportion score was assigned, which represented the estimated proportion of positive-staining tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3; to 2/3; and 5, >2/3). Next, an intensity score was assigned, which represented the average intensity of

positive tumor cells (0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. Tumour was defined as ER/PR positive if their total IHC score was greater than 2 and ER/PR negative if their score was 0 or 2 (Allred and Berardo, 1998). Thus, the total score of 3, the lowest possible positive score, corresponds to as few as 1% to 10% weakly staining tumor cells. The cut-off point of positivity when a total IHC score of greater than 2 is very significant as it had a significantly improved response, compared with those who had lower scores (Harvey et al., 1999).

HER2 scoring

HER2 was scored as 0, 1+ and 2+ and 3+. HER2 status was considered as negative or non-overexpression if IHC score was 0 or 1+, borderline reactivity if the score was 2+, and positive if the score was 3+. Score 0; No reactivity or membranous reactivity in <10% of tumour cells. Score1; Faint/barely perceptible membranous reactivity is detected in >10% of tumour cells. The cells are immunoreactive only in part of the membrane. Score 2; Weak to moderate complete membranous reactivity is seen in >10% of tumour cells. Score 3; Strong complete reactivity is seen in >10% of tumour cells (Ellis and Bartlett, 2004; Ambroise et al., 2011). Borderline 2+ samples require confirmation using another analysis method, ideally FISH but we used chromogenic in-situ hybridization (CISH) as an alternative of Fluorescent in-situ hybridization (FISH).

The CISH hybridizations were evaluated using a light microscope. Unaltered gene copy number was defined as 1 to 5 signals per nucleus. Low-level amplification was defined as 6 to 10 signals per nucleus in 50% of cancer cells, or when a small gene copy cluster was found. Amplification of HER2 was defined when a large gene copy cluster in 50% of carcinoma cells or numerous (>10) separate gene copies seen (Tanner et al., 2000).

Statistical analysis

All cases were analysed after successful optimization of the immunohistochemical method and agreement of the scoring system. The evaluation and scoring were done under 400X magnification. The scoring was done based on the microscopic analysis as described above. Data was entered and statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS)

Table 1. Association between PITX2 Expressions with Clinicopathological Data

		PITX2 Expression				
		category 1		category 2		
		n	(%)	n	(%)	p
Age	≤50 years	26	(50.0)	26	(50.0)	0.523
	>50 years	21	(43.8)	27	(56.3)	
Race	Malay	38	(50.0)	38	(50.0)	0.285
	Non-Malay	9	(37.5)	15	(62.5)	
Tumor size	≤2 cm	5	(50.0)	5	(50.0)	0.896
	>2 cm but ≤5 cm	20	(44.4)	25	(55.6)	
	>5 cm	22	(48.9)	23	(51.1)	
Tumor grading						
Nuclear pleomorphism	Score 1	3	(42.9)	4	(57.1)	0.715
	Score 2	30	(44.8)	37	(55.2)	
	Score 3	14	(53.8)	12	(46.2)	
Mitotic count	Score 1	13	(46.4)	15	(53.6)	0.435
	Score 2	7	(35.0)	13	(65.0)	
	Score 3	27	(51.9)	25	(48.1)	
Glandular formation	Score 1	4	(50.0)	4	(50.0)	0.044
	Score 2	8	(27.6)	21	(72.4)	
	Score 3	35	(55.6)	28	(44.4)	
Tumor grade	Grade 1 (total=3-5)	8	(50.0)	8	(50.0)	0.382
	Grade 2 (total=6-7)	15	(38.5)	24	(61.5)	
	Grade 3 (total=8-9)	24	(53.3)	21	(46.7)	
Lymph node metastasis	Negative	21	(44.7)	26	(55.3)	0.662
	Positive	26	(49.1)	27	(50.9)	
Lymph node metastasis	0 node	21	(44.7)	26	(55.3)	0.451
	1 to 3 nodes	16	(59.3)	11	(40.7)	
	4 to 9 nodes	7	(41.2)	10	(58.8)	
	>10 nodes	3	(33.3)	6	(66.7)	
Estrogen receptor	Negative	28	(62.2)	17	(37.8)	0.006
	Positive	19	(34.5)	36	(65.5)	
Progestron receptor	Negative	28	(66.7)	14	(33.3)	0.001
	Positive	19	(32.8)	39	(67.2)	
HER2 receptor	Negative	34	(47.2)	38	(52.8)	0.943
	Positive	13	(46.4)	15	(53.6)	
Molecular subtypes	HER2-ER-PR-	16	(59.3)	11	(40.7)	0.051
	HER2-ER&PR+	18	(40.0)	27	(60.0)	
	HER2+ER&PR+	6	(31.6)	13	(68.4)	
	HER2+ER-PR-	7	(77.8)	2	(22.2)	

programmed version 18.0. The association between PITX2 expression with growth receptors ER, PR and HER2 and also other clinicopathological variables status were determined using the chi-square test. Associations were considered statistically significant when p value equal or less than 0.05.

Ethical Approval: This study protocol was approved by Research Ethics Committee (Human), Universiti Sains Malaysia. Approval for using archive tissue blocks were obtained from the head of department and hospital director.

Results

A total of 100 cases were included in this study. 84 cases were from mastectomy specimen, 9 cases from wide excision and 7 cases were lumpectomy specimen. The youngest age was 28 years while the oldest was 89 years with the mean age of 50.5 years. 99 cases were female and only 1 was a male breast.

Nearly half of the patients had tumour size range from 2 to 5 cm (45%) and another 45% had tumour size of more than 5 cm. The largest tumour size in this sample was 22 cm, the smallest was 0.9 cm and the mean tumour size was 6 cm. Majority of the cases showed moderate nuclear pleomorphism with score of 2 (67%). Approximately half of the patients with invasive ductal carcinoma (IDC) in this study were grade III (45%) followed by grade II (39%) and grade 1 (16%). Out of 53 of cases, 27% of them showed only 1 to 3 nodes involvement, 17% involved 4 to 9 lymph nodes and only 9% had metastases to more than 10 lymph nodes. In addition, more than half of the cases showed immunoreactivity for estrogen receptor (55%) and progesterone receptor (58%). Meanwhile, 45% of the cases showed molecular subtypes of HER2-ER&/PR+, followed by triple negative groups (27%), molecular subtypes of HER2+ER&/PR+ (19%) and HER2+ER&PR- (9%).

Expression of PITX2 was determined by using semiquantitative score based on percentage of tumour cells picked up the stain and the staining intensity in 100 cells count. Based on the histogram curve of cytoplasmic

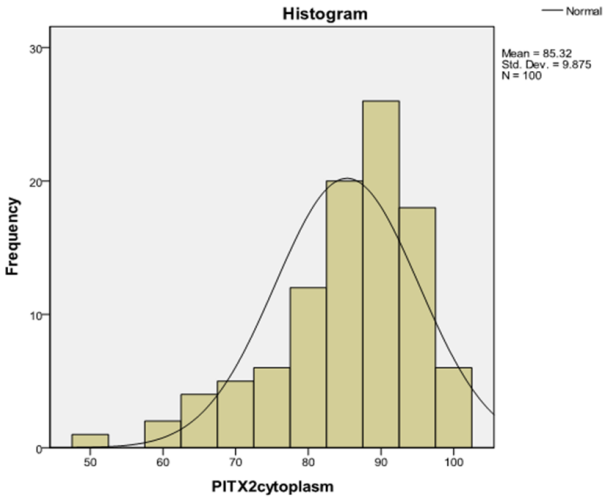

Figure 1. Histogram of Cytoplasmic Immunoreactivity of PITX2

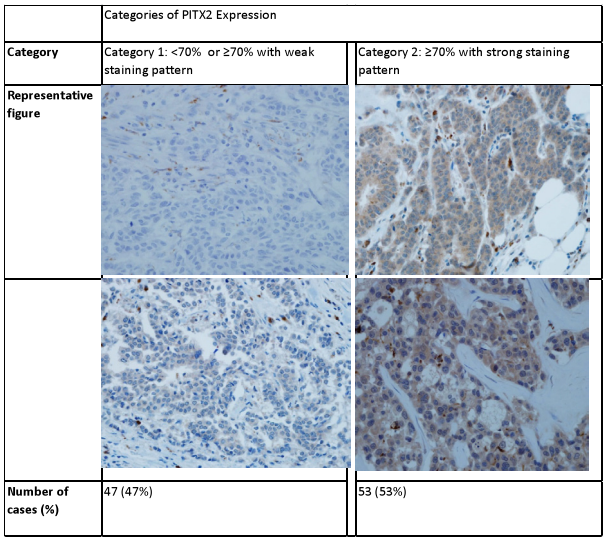

Figure 2. The Categories of PITX2 Expression with their Description

Table 2. Multivariate Analysis for Clinicopathologic Parameters with PITX2 Expression

Clinicopathological data	RR	95% CI	p value
ER	3.121	1.375-7.084	0.005
PR	4.105	1.765-9.547	0.001

immunoreactivity, the cut-off point ≥70% cells were taken to determine the positivity (Figure 1). The expression of PITX2 was divided into 2 categories; category 1: <70% or ≥70% cells with weak staining pattern and category 2: ≥70% with strong staining pattern (Figure 2). The expression of PITX2 in invasive breast carcinoma was determined to be 53%.

Discussion

The mean age of patients at the time of breast cancer diagnosis in this study was 50.5 which was more or less similar to other recent studies done by Hasnan et al (2012) in Malaysia, Zubeda et al (2013) in India and Li et al (2013) in China. From this study we found that the percentage of patients with phenotype triple negative breast cancer was 27% which was higher than Chinese patients (17%) (Ma et al., 2012), Turkish patients (10-15%) (Somali et al., 2013) but lower than Indian patients (46%) (Zubeda et al., 2013).

Recent literatures have introduced a paired-like homeodomain transcription factor 2 (PITX2) as a DNA methylation marker that enable to predict the outcome in breast cancer. A study by Widschwendter et al (2008) mentioned that PITX2 is one of the genes showing higher methylation in breast cancer. Following that, several other studies were done to validate the methylation status of PITX2 by using cell line and Quantitative DNA-methylation PCR (QM-PCR) specific on fresh frozen tissue as well as formalin-fixed tissue. They found that there was hypermethylation of PITX2 at P2 regulatory promoter site of CpG island in breast carcinoma that cause reduction in gene expression and this was associated with poor prognosis (Nimrich et al., 2008; Maier et al., 2007;

Harbeck et al., 2008).

Hypermethylation at P2 promoter of this gene was thought to be associated with poor prognostic factor as it shortened the time of distant metastasis and disease recurrent. Our present study was carried out with the aim to determine the expression of PITX2 protein in invasive ductal carcinoma and its association with the established growth receptors such as ER, PR and HER2. By determining their association, the role of PITX2 in breast carcinoma can be revealed as another potential prognostic marker in breast cancer.

Through this study, we were able to provide good evidence that PITX2 plays a role in breast carcinoma. We found that there was 53% expression of PITX2 protein by IHC in invasive ductal carcinoma and lack of PITX2 protein expression in 47% of cases. The immunoreactivity of PITX2 protein was in the cytoplasm and not in the nucleus.

Univariate analysis revealed a significant association between PITX2 expression with ER (0.006), PR (p=0.001), gland formation (p=0.044) and molecular subtypes (marginally associated p=0.051). Significant association was also found between combined expressions of ER and PR with PITX2 (p=0.003), especially for the double positive ER and PR (ER+PR+). Association of PITX2 with these factors proven that PITX2 expression is able to serve as a good prognostic factor.

Multivariate analysis showed that PITX2 and PR were the most significantly associated (RR 4.105, 95%CI 1.765-9.547, p=0.001) as compared to other factors (Table 2). This result conferred a new finding emphasized on the important of PR positivity in PITX2 positive cases to be a good prognostic marker. In the case of PR negative eventhough ER and PITX2 were positive, the prognosis might be poorer.

All the above findings were in agreement with previous studies on DNA methylation of PITX2 which have influenced the prognosis of breast carcinoma. Eventhough the methylation cannot be directly measured by immunohistochemistry method, but the concept of hypermethylation of the gene that lead to transcriptional silencing resulting in reduction of protein expression can be demonstrated. Thus, lack of PITX2 protein expression represents hypermethylation of PITX2 gene but high expression of PITX2 protein indicates no methylation occurred.

In conclusion, we demonstrated the PITX2 is another potential prognostic marker in breast carcinoma that able to add significant information to established prognostic factors of ER and PR. Based on the result, we proposed the use of expression PITX2 together with PR may carry a very good prognosis.

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