Tumor budding in breast carcinoma: relation to E-Cadherin, MMP-9 expression, and metastasis risk

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ABSTRACT

Background: Tumor budding is a histopathologic entity refers to small cluster of cancer cells at the invasive edge of tumor. It was assumed that tumor budding is linked to epithelial-mesenchymal transition, an early event in metastasis.
Objective: This study aimed to find out the correlation of tumor budding with E-cadherin and MMP-9 expression and risk of metastasis in breast carcinoma.
Method: We investigated 35 cases breast carcinoma with metastasis and 35 cases without metastasis. The number of tumor budding was counted in cytokeratin-stained slides with 400x magnification ($0.57~mm^2$).

Result: Cut-off point by ROC analysis was 11 and the patient was categorized into low grade (0-10 buds) and high grade (11 or more buds) tumor budding. Inter-observer agreement was good with K value 0.914. Low level of E-cadherin was not significantly correlated with high grade tumor budding (p=0.660), meanwhile high level of MMP-9 was significantly correlated with high grade tumor budding (p=0.001). High grade tumor budding was a significant, independent risk factor of metastasis in breast carcinoma (OR=38.2, 95% CI 7.5-193.7, p<0.001).
Conclusion: In conclusion, tumor budding grade is related to level of MMP-9 but has no correlation E-cadherin expression. High grade tumor budding is an independent risk factor of metastasis in breast carcinoma.

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INTRODUCTION

Distance metastasis is the most common cause of death in breast carcinoma patient. Many prognostic factors, clinicopathological to molecular factors, had been investigated and applied to daily clinical practice of breast cancer patient. New markers were still in research, one of them was tumor budding. Tumor budding is a small cluster of cancer cells (1-5 cells) at the periphery of tumor.[1,2] Tumor budding hypothetically assumed as an epithelial-mesenchymal transition manifestation (EMT)[2], an early change in metastatic cascade. EMT is characterized by decreased of E-cadherin expression and increased MMP-9 expression.[3,4,5]

In this study, we try to determine whether low level of E-cadherin and high level of MMP-9 expression were correlated with high grade tumor budding, and to investigate high grade tumor budding as a risk factor of metastasis in breast carcinoma.

METHODS

This study was a cross sectional and nested case control study of breast cancer patients at Sanglah Hospital and Prima Medika Hospital from 1 January 2012 until 30 June 2015. Thirty-five patients presenting with invasive carcinoma of no special type with metastasis and 35 patients without

metastasis and had undergone regular follow-up with chest x-ray and abdominal ultrasound were studied. Clinicopathological data included age, tumor size, nodal status, stage, histological tumor type, grade, tumor infiltrating leucocytes (TIL) and lymphovascular invasion (LVI).

The data was from medical record and histopathology reports. Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki-67 Immunohistochemical analysis were from immunohistochemistry reports. The cut-off points of ER and PR were 1%[6] and Ki-67 was 14%.[6,7] HER2 was reported according WHO guidelines[6] and 3+ staining was considered positive.

Immunostaining

The selected paraffin blocks were sliced into 4 mm thickness to performed immunostaining. The section was stained with mouse anti-human cytokeratin (AE1/AE3, Dako, Denmark), mouse anti-human E-cadherin (NCH-38, Dako, Denmark) and mouse anti-human MMP-9 (EP1255Y, Abcam, USA) as primary antibodies. Endogenous peroxidase enzyme was blocked with 0.3% hydrogen peroxide for 20 minutes. Heat–induced epitope, retrieved in citric buffer for 20 minutes as antigen retrieving. Slides were incubated with the antibodies for 2 hours. Negative controls were carried out by skipping primary

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antibodies. Immunodetections were done with Starr Trek Universal HRP Detection (Lab Vision, USA).

Evaluation of tumor budding

Tumor budding was defined as small cluster (1-5) tumor cells at the invasive front.[1,2] The number of tumor budding was counted in cytokeratin-stained slides. For each case, tumor budding was counted in five densest budding using an Olympus microscope (CX-41, 400x, field size 0.57 mm^2). The number of tumor budding was the highest count per case (Fig. 1A and 1B). According to cut-off point determined by receiver operating curve (ROC) analysis, the cases categorized into low grade and high grade tumor budding. Inter-observer agreement was tested between two independent observers (NPS and IGAA). Discordances between two independent observers were resolved by simultaneous review and this data was used to do further statistical analysis.

Evaluation of E-cadherin and MMP-9 expression

Immunostaining status of E-cadherin was determined by counting the membranous staining cancer cells. More than 10% staining cells considered high expression of E-cadherin[8]. The MMP-9 staining status was determined by counting the cytoplasm staining cancer cells. More than 10% staining cells considered high expression of MMP-9.[9]

Statistical analysis

The cut-off for tumor budding was determined by ROC analysis. Inter-observer agreement was tested by kappa test. Correlation between E-cadherin and MMP-9 and grade of tumor budding were determined by chi-square test. Correlation between high grade tumor budding and metastasis, and between clinicopathological characteristic and metastasis, was tested by chi-square test, odd ratio, and logistic regression, with $p<0.05$ significance. Analysis was done using STATA software 12.0 (Statacorp, USA).

RESULTS

In the present study, the results of various studies analyzed based on indicators of systemic assessment of Baartman and Vander Vlutenstudies[47-49] Validity (Fit for purpose) and Reliability (Reproducibility of decisions) are two major criteria for evaluation system.

From 70 patients, mean age was 48.6 years (range 23-74 years). The sites of metastasis in metastasis group are shown in Fig. 2. In non-metastasis group, the mean duration of follow-up was 27.4 months (range 14-40 months). Clinicopathological characteristics of the patients (n=70) are shown in table 1.

Tumor budding

The range of actual number of tumor buds was from 2 to 40. By ROC analysis, 11 buds (0.57 mm^2 size field) were selected as the cut-off point (77.14% for sensitivity and 85.71% for specificity). Then, cases were classified into low grade (0-10 buds, 38 cases, 54.29%) and high grade (11 or more buds, 32 cases, 45.71%) tumor budding. Inter-observer agreement was good with K value = 0.914.

Correlation between E-cadherin expression and tumor budding

E-cadherin expression was compared with grade of tumor budding. Most of the patients showed high level of E-cadherin expression, and observed in groups, low grade and high grade tumor budding groups. Low level of E-cadherin was not significantly correlated with high grade tumor budding (p=0.660) (Table 2).

Correlation between MMP-9 expression and tumor budding

MMP-9 expression was compared with grade of tumor budding. Although most of the patients showed high level of MMP-8 expression, and also

Table 1 The clinicopathological characteristics of patients (n=70)

Characteristics	Patients (n)	Percentage (%)
Age (≤ 40/>40 years)	15/55	21.43/78.57
Size (T1-T2/T3-T4)	23/47	32.86/67.14
Nodal status (-/+)	8/62	11.43/88,57
Stage (I-II/III-IV)	19/51	27.14/72.86
Histology grade (1-2/3)	35/35	50/50
TIL (mild/severe)	51/19	72.86/27.14
LVI (-/+)	17/53	24.29/75.71
Ki-67 (low/high)	7/63	10/90
ER (ER-/ER+)	30/40	42.86/57.14
PR (PR-/PR+)	31/39	44.29/55.71
HER2 (HER2-/HER2+)	57/13	81.43/18.57

Table 2 Correlation between E-cadherin expression and tumor budding grade

E-cadherin	Tumor budding		P value
	LG TB (n = 38)	HG TB (n = 32)	
Low	2 (5.26%)	1 (3.13%)	0.660
High	36 (94.74%)	31 (96.88%)	

LG TB: *low grade tumor budding*, HG TB *high grade tumor budding*

observed in both groups, low grade and high grade tumor budding groups, but high level of MMP-9 was correlated with high grade tumor budding (p=0.001) (Table 3).

Table 3 Correlation between MMP-9 expression and tumor budding grade

| MMP-9 | Tumor budding | | P value |
	LG TB (n = 38)	HG TB (n = 32)	
Low	11 (28.95%)	0 (0%)	<0.001
High	27 (71.05%)	32 (100%)	

LG TB: *low grade tumor budding*, HG TB *high grade tumor budding*

Table 4 The relationship between high grade tumor budding and metastasis, and between clinicopathological characteristics and metastasis

| | Univariate analysis | | Multivariate analysis | |
	OR (95% CI)	P value	OR (95% CI)	P value
Tumor budding (LG/HG)	20.25 (5.2-85.6)	<0.001	38.2 (7.5-193.7)	<0.001
Age (≤ 40/>40 years)		0.328		
Size (T1-T2/T3-T4)	57.73 (7.3-2441.4)	<0.001		
Nodal status (-/+)		0.003		
Stage (I-II/III-IV)		<0.001		
Histology grade (1-2/3)	2.9 (1.0-8.5)	0.031	7.6 (1.5-37.8)	0.013
TIL (mild/severe)	1.6 (0.4-3.8)	0.788		
LVI (-/+)		<0.001		
Ki-67 (low/high)		0.005		
ER (ER-/ER+)	0.6 (0.2-1.8)	0.334		
PR (PR-/PR+)	1.1 (0.4-3.2)	0.810		
HER2 (HER2-/HER2+)		0.759		

L G: low grade, HG high grade
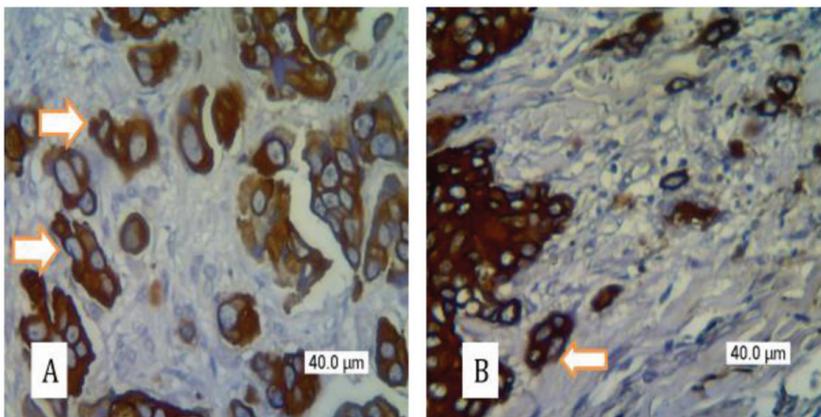
Figure 1 *Tumor budding* is a small cluster (1 to 5) tumor cells in invasive edge of tumor, evaluated in cytokeratin-stained slides and categorized into *high grade* (≥11 *tumor budding*) (A) and *low grade* (0-10 *tumor budding*) (B). (400×)

High grade tumor budding and risk of metastasis in breast carcinoma

The relationship between high grade tumor budding and metastasis, and between clinicopathological characteristics and metastasis are shown in Table 4. Univariate analysis showed high grade tumor budding (OR 20.25, 95% CI 5.2-85.6 P<0.001), large tumor size (OR 57.53, 95% CI 7.32-2441.4, P<0.001) and histology grade 3 (OR 2.9, 95% CI 0.98-8.46, P=0.031) were statistically significant correlated with metastasis. Multivariate analysis showed high grade tumor budding (OR=38.2, 95% CI 7.5-193.7, P<0.001) and histology grade 3 (OR=7.6, 95% CI 1.5-37.8, P<0.001) were significantly related to metastasis.

DISCUSSION

Various clinicopathological and molecular parameters have been proved as prognostic factors in patient with breast carcinoma. Tumor budding is a new marker in breast carcinoma. There were many studies about tumor budding, especially in colorectal carcinoma. High grade tumor budding was significantly correlated with bad prognosis. UICC, in colorectal cancer, has been including the evaluation of tumor budding, in addition to standard microscopic parameters.[10]

Tumor budding is one of mechanisms of cancer invasion and metastasis. Cancer with high grade tumor budding shares aggressive molecular and biological characteristics. Tumor budding is considered as a representation of EMT.[2] Decrease of E-cadherin and increase of MMP-9 expression are some markers of EMT.[3,4,5] E-cadherin is an adhesion molecule of epithelial cells. In many cancers, loss of E-cadherin expression of tumor cells is found in poorly differentiated tumor. In breast carcinoma, loss of E-cadherin expression is mainly found in invasive lobular carcinoma and this immunostaining is performed if there is a difficulty in differentiating invasive ductal and lobular type carcinoma. In lobular carcinoma, genetic and epigenetic alteration in gene encoding E-cadherin results in loss of E-cadherin protein expression, even found at an early stage (in situ) tumors.[8,11] In this study, we only used invasive carcinoma of no special type cases and high level of E-cadherin expression was found in both of groups, low grade (36 of 38, 94.74%) and high grade (31 of 32, 96.88%) tumor budding.

Tumor budding grade was not significantly associated with low level of E-cadherin expression. Previous study showed E-cadherin expression in tumor budding area was higher compared with in the central area of the tumor.[12] Different result is possibly due to different criteria and method.

Studies that assess the correlation between the expression of E-cadherin with other prognostic parameters, as well as the aggressiveness of the tumor markers, showed inconsistent results.[8,13-17]

MMP-9 is a zinc-dependent endopeptidase which is important in degradation of type IV collagen, a component of basal membrane. In EMT, there is an increase of MMP-9 expression. In this study, there was a significant relationship between high-grade tumor budding and high level of MMP-9 expression. Several studies of the expression of MMP-9 in breast cancer have been performed, but there was no study about the correlation between expressions of MMP-9 with tumor budding grade in breast carcinoma previously. Many studies about correlation between MMP-9 expression and clinicopathological characteristics and prognosis have been done. Increased MMP-9 expression is related to histology grade, stage, tumor type, lymphatic invasion and nodal metastasis.[9,18,19,20] Breast carcinoma patients with positive expression of MMP-9 showed a high risk of relapse, metastasis and poor survival.[19,20]

The expression of MMP-9 varies among molecular subtypes of breast carcinoma, and over-expression of MMP-9 is a marker of TNBC and HER2-positive breast cancer.[20] High grade tumor budding reflects progression of cancer.[21] Several studies have shown prognostic value of tumor budding in several malignancies, including in colorectal carcinoma,[22] breast carcinoma,[12] and another epithelial tumor.

In this study, high histology grade and high grade tumor budding was independently correlated with metastasis in breast carcinoma. There was no previous studies that examined the correlation between tumor budding and distance metastasis in breast carcinoma. Previous study about tumor budding in breast carcinoma showed correlation between tumor budding with several clinicopathologic factors, risk of nodal metastasis and survival in breast cancer patients.[12,23,24]

CONCLUSION

Tumor budding in breast carcinoma patient shows partial feature of EMT. High grade tumor budding is an independent risk factor of metastasis in breast carcinoma.

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