Expression of vascular endothelial growth factor and caspase-3 in mucinous breast carcinoma and infiltrating ductal carcinoma-not otherwise specified, and the correlation with disease-free survival

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Abstract. Mucinous breast carcinoma (MBC) is a rare type of breast cancer, but it has been infrequently studied due to its associated good prognosis. Vascular endothelial growth factor (VEGF) and caspase-3 have been identified to be prognostic factors of infiltrating ductal carcinoma-not otherwise specified (IDC-NOS), but their expression in MBC has not been reported. In the present study, the expression of caspase-3 and VEGF in MBC and IDC-NOS were assessed by immunohistochemistry. Scoring was conducted based on staining intensity and percentage of positive cells. Based on the scores of caspase-3 and VEGF expression, all patient samples were divided into two groups: Low expression (score of 0-5) or high expression (score of 6-12). In total, 42.59% of MBC patients exhibited a high VEGF score compared with 61.67% of the IDC-NOS group ($P<0.05$). Furthermore, 57.41% of MBC patients exhibited high caspase-3 expression compared with only 33.33% of IDC-NOS patients ($P<0.05$). VEGF expression in MBC was associated with age, nodal status and tumor-node-metastasis (TNM) stage. Cox univariate analysis showed that higher VEGF expression, positive nodal status and higher TNM stage were associated with shorter disease-free survival (DFS). The Kaplan-Meier method showed that higher VEGF expression in MBC was associated with worse DFS times, while Cox multivariate analysis showed that only TNM stage was significantly associated with DFS. VEGF and caspase-3 expression varied in the MBC and IDC-NOS samples, but neither was directly correlated with DFS in the MBC patients.

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Introduction

Mucinous breast carcinoma (MBC), also known as colloid carcinoma, is a rare subtype of breast tumors that accounts for 1-7% of all breast cancer cases. MBC is characterized by the presence of extracellular mucin (MUC) (1). MBC includes mixed MBC, consisting of other cancer types such as invasive ductal carcinoma, and pure MBC (PMBC), in which the entire mass is almost occupied by mucinous cancer cells and is without conventional invasive ductal carcinoma cells (2). PMBC is represented by a mass with a >90% mucinous component (3). MBC is linked with a more favorable prognosis, a longer disease-free interval and a lower incidence of axillary node metastasis compared with infiltrating ductal carcinoma-not otherwise specified (IDC-NOS) (1,2,4). However, recurrence and metastasis of MBC are frequently present in clinical practice.

Angiogenesis is a prerequisite for tumor development; there is a close association between the formation of blood vessels in the vicinity of tumor cells and the potential for tumor formation, invasion and metastasis. Angiogenesis is induced and developed in response to two sets of extracellular signals: soluble angiogenic factors and the extracellular matrix (5). Breast carcinoma has been shown to be an angiogenesis-dependent tumor through experimental and clinical data. Vascular endothelial growth factor (VEGF) is the most potent endothelial cell mitogen (6) and a regulator of vascular permeability, therefore, VEGF has been considered as a powerful novel prognostic tool (7). However, the associations between VEGF expression in MBC and IDC-NOS and the morphology, behavior and prognosis of tumors, and the differences between MBC and IDC-NOS, are unclear.

The good prognosis of MBC is closely associated with the formation of MUC around the cells (8). Previous studies have revealed the expression of the MUC1, MUC2, MUC3, MUC4, MUC5A and MUC6 proteins in PMBC, and this expression has been suggested to be a prognostic factor (9). Gel-forming secretory MUCs, including MUC2 and MUC6, exhibit a high expression rate in mucinous carcinoma, indicating that high production of these types of MUCs may act as a barrier to the extension of cancer, resulting in less aggressive biological behavior. However, the expression of

MUC1, which is associated with a poor prognosis in gastric and colorectal cancer types, is low in MBC (9). A study by Ahmed (10) highlighted that the MUC of MBC is derived from cell breakdown. We hypothesize that significant cell apoptosis may exist in the MBC tissues and produce a large amount of mucus. The most well-known biochemical hallmark of early- and late-stage apoptosis is cysteine protease activation. Arginine-glycine-aspartate synthetic peptides induce apoptosis by direct caspase-3 activation (11). Caspase-3 is also required for the DNA fragmentation and morphological changes associated with apoptosis (12). A high level of active caspase-3 in cells and tissues is an important biomarker for apoptosis induced by a wide variety of apoptotic signals (13). Thus, detection of caspase-3 expression can reflect the apoptotic status of tumor cells, which may aid in explaining the differences in prognosis and survival between MBC and IDC-NOS.

In the present study, the expression of VEGF and caspase-3 in MBC and IDC-NOS was investigated using immunohistochemical staining, and the association between the expression levels of VEGF and caspase-3 and clinicopathological features were further investigated.

Materials and methods

Patients and tissues. A total of 54 patients with MBC and 60 randomly selected patients with IDC-NOS who underwent surgery at the First Affiliated Hospital of China Medical University (Shenyang, Liaoning, China) between May 2009 and June 2011 were included in the present study. MBC is a rare type of breast cancer, so the 54 patients with MBC included all the MBC in accordance with the set of conditions between May 2009 and June 2011 who underwent surgery at the First Affiliated Hospital of China Medical University. Cases in which complete clinicopathological information and formalin-fixed, paraffin-embedded breast tissues could not be obtained were excluded. All patients had not received radiotherapy or chemotherapy prior to surgery. The diagnosis of all cases was confirmed according to the criteria of the World Health Organization (14), as assessed by the Department of Pathology (First Affiliated Hospital). Archival formalin-fixed paraffin-embedded breast tissues were retrieved. Patients were followed up for a median period of 47 months (range, 28-77 months) subsequent to the initial cancer surgery. Follow-up consisted of regular clinic visits and ultrasound of the breast and axillary node, supraclavicular area and infraclavicular region, with or without mammography, lung computed tomography, liver ultrasound, bone emission computed tomography and blood tests at the discretion of the treating specialist. Relevant clinical and pathological information are described in Table I.

Immunohistochemical staining. Immunohistochemical examination was performed on 4-μm thick, formalin-fixed, paraffin-embedded sections using UltraSensitive™ SP IHC kit (MXB Co., Ltd., Fuzhou, China). Briefly, following deparaffinization (with xylene) and rehydration (with alcohol), the endogenous peroxidase activity was blocked with 3% H_2O_2. Antigen retrieval was performed with a high-pressure cooker and normal serum (part of the UltraSensitive™ SP IHC kit) was applied to the sections at room temperature for 30 min to block non-specific antibody binding. The sections were then incubated overnight at 4˚C with the primary antibodies, including monoclonal mouse-anti-human caspase-3 (1:50 dilution; catalog no. ab2171; Abcam, Cambridge, MA, USA) and monoclonal mouse-anti-human VEGF (1:300 dilution; catalog no. sc-7269; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Sections were further incubated with the biotin-labeled IgG secondary antibody solution from the UltraSensitive™ SP IHC kit at room temperature for 15 min, followed by streptavidin-peroxidase incubation at room temperature for 15 min. Finally, sections were stained with 3,3-diaminobenzidine, counterstained with hematoxylin for 5 min and mounted. Negative controls were processed with PBS instead of the primary antibody.

Immunohistochemical scoring. The immunostained sections were assessed with an optical microscope at x400 magnification, based on manual counting of positive cells in each tissue by two observers blinded to clinical outcomes. Cases of disagreement were reviewed jointly to obtain a consensus score. The percentage of positive cells and staining intensity of VEGF and caspase-3 were scored. Intensity was graded as negative (score 0), weak (score 1), moderate (score 2) or strong (score 3), and percentage of positive cells was graded as <5% (score 0), 5-25% (score 1), 26-50% (score 2), 51-75% (score 3) and >75% (score 4). The final score of VEGF and caspase-3 expression was determined by multiplying the intensity score and percentage score, with a range of 0-12. According to the scoring results, all patients were divided to two groups: Low (score of 0-5) and high (score of 6-12) expression.

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 statistical software (IBM Corp., Armonk, New York, USA). A χ^2 test and Fisher's exact test were used to identify the differences between MBC and IDC-NOS with regard to clinicopathological features, and for the associations between VEGF or caspase-3 and clinicopathological variables. Disease-free survival (DFS) was recorded from the date of surgery to the relapse date or the last follow-up date, and was estimated using the Kaplan-Meier analysis. The statistical significance of differential survival was assessed using the log-rank test. Cox regression analysis for DFS was used. $P<0.05$ was used to indicate a statistically significant difference. Variables with a univariate p-value of <0.1 were included in the multivariate model.

Results

Characteristics of the study population. A total of 54 female patients with MBC (median age 53.87 years; range, 24-82 years) and 60 IDC-NOS (median age 50.08 years; range, 27-78 years) were included. The cohort consisted of 94.5% pathological tumor stage 1-2 (pT_{1-2}) patients, 5.6% pT_{3-4} patients and 16.8% node-positive patients in the MBC group, and 98.4% pT_{1-2} patients, 1.7% pT_{3-4} patients and 45.0% node-positive patients in the IDC-NOS group. No metastasis was observed in either group prior to surgery. In the MBC group, 92.6% of patients chose to undergo a mastectomy and 7.4% of patients chose breast-conserving

Table I. Clinical and pathological features of the patients (n=114).

Characteristics	MBC (n=54)	IDC (n=60)	P-value
Age, years			
Mean	53.87	50.08	0.079
≤50, n (%)	26 (48.15)	35 (58.33)	0.276
>50, n (%)	28 (51.85)	25 (41.67)	
T stage, n (%)			0.203
pT1	23 (42.59)	19 (31.67)	
pT2	28 (51.85)	40 (66.67)	
pT3-4	3 (5.56)	1 (1.67)	
N stage, n (%)			0.010[a]
N0	45 (83.33)	33 (55.00)	
N1	5 (9.26)	15 (25.00)	
N2	3 (5.56)	7 (11.67)	
N3	1 (1.85)	5 (8.33)	
TNM stage, n (%)			0.023[a]
I	23 (42.59)	13 (21.67)	
II	27 (50.00)	35 (58.33)	
III	4 (7.41)	12 (20.00)	
ER status, n (%)			0.001[a]
Negative	6 (11.11)	23 (38.33)	
Positive	48 (88.89)	37 (61.67)	
PR status, n (%)			0.147
Negative	18 (33.33)	28 (46.67)	
Positive	36 (66.67)	32 (53.33)	
Hormone receptor status, n (%)			0.001[a]
Negative	5 (9.26)	22 (36.67)	
Positive	49 (90.74)	38 (63.33)	
HER-2 status, n (%)			0.085
Negative	1 (1.85)	35 (58.33)	
Positive	43 (79.63)	10 (16.67)	
Unknown	10 (18.52)	15 (25.00)	
Ki-67, n (%)			0.004[a]
≤20%	41 (75.93)	30 (50.00)	
>20%	13 (24.07)	30 (50.00)	
p53, n (%)			0.883
Negative	19 (35.19)	22 (36.37)	
Positive	28 (51.85)	32 (53.33)	
Unknown	7 (12.96)	6 (10.00)	
Surgery			0.420
Mastectomy	50 (92.59)	58 (96.67)	
BCS	4 (7.41)	2 (3.33)	
Axillary operation			0.045[a]
Sentinel lymph node biopsy	8 (14.81)	2 (3.33)	
Axillary clearance	46 (85.19)	58 (96.67)	
Chemotherapy			<0.001[a]
No	12 (22.22)	2 (3.33)	
Yes	42 (77.78)	58 (96.67)	
Anthracycline included	31 (57.41)	13 (21.67)	
Taxane included	4 (7.41)	3 (5.00)	
Anthracycline and taxane included	6 (11.11)	41 (68.33)	
Other	1 (1.85)	1 (1.67)	
Radiotherapy			0.12
No	54 (100.00)	56 (93.33)	
Yes	0 (0.00)	4 (6.67)	

[a]Significant difference between MBC and IDC-NOS (P<0.05). MBC, mucinous breast carcinoma; IDC-NOS, infiltrating ductal carcinoma-not otherwise specified; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor-node-Metastasis; BCS, breast-conserving surgery; HER2, human epidermal growth factor receptor 2; p53, cellular tumor antigen p53.

Table II. VEGF and caspase-3 expression in MBC and IDC-NOS.

Expression	MBC, n (%)	IDC-NOS, n (%)	P-value
VEGF high	23 (42.59)	37 (61.67)	0.042[a]
Caspase-3 high	31 (57.41)	20 (33.33)	0.010[a]

[a]Significant difference between MBC and IDC-NOS (P<0.05). MBC, mucinous breast carcinoma; IDC-NOS, infiltrating ductal carcinoma-not otherwise specified; VEGF, vascular endothelial growth factor.

surgery. Furthermore, 14.8% of patients underwent sentinel lymph node biopsy and 85.2% of patients received axillary clearance. In the MBC group, 22.2% of patients did not accept adjuvant chemotherapy and no patients accepted adjuvant radiotherapy following surgery. There were no significant differences in terms of age, T stage, breast surgery and adjuvant radiotherapy between the two groups. In contrast to IDC-NOS, the MBC patients showed a higher rate of positive ER and hormone receptor, and a larger population of which expression Ki-67 was ≤20%. MBC patients tended to have significantly less lymph node metastasis and a lower tumor-node-metastasis (TNM) stage (15) compared with IDC-NOS patients (P=0.010 and P=0.023, respectively) (Table I).

VEGF and caspase-3 expression in MBC and IDC-NOS patients. The positive staining of VEGF and caspase-3 was mainly observed in the cytoplasm (Fig. 1). The expression of VEGF and caspase-3 was significantly different between the MBC and IDC-NOS patients. In total, 42.59% of MBC patients exhibited a high VEGF score (≥6), with this

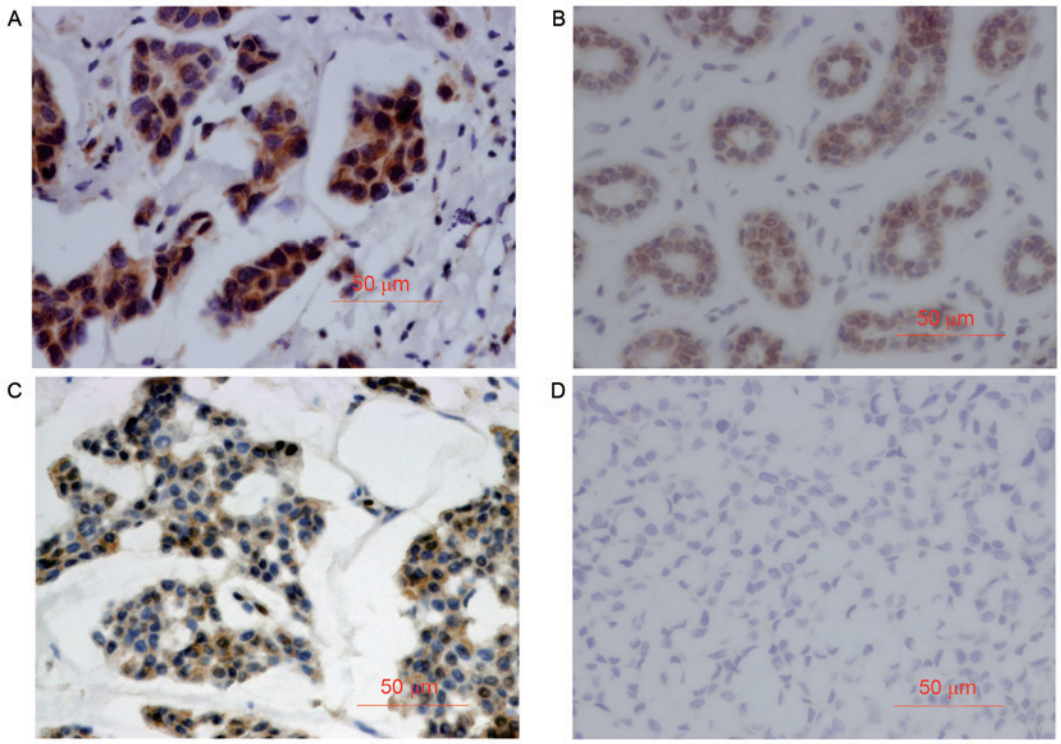

Figure 1. Expression of VEGF and caspase-3 in MBC and IDC-NOS. (A) The high expression of VEGF in MBC. (B) High expression of VEGF in IDC-NOS. (C) The high expression of caspase-3 in MBC. (D) The low expression of caspase-3 in IDC-NOS. Scale bar, 50 μm; original magnification, x400. VEGF, vascular endothelial growth factor; MBC, mucinous breast carcinoma.

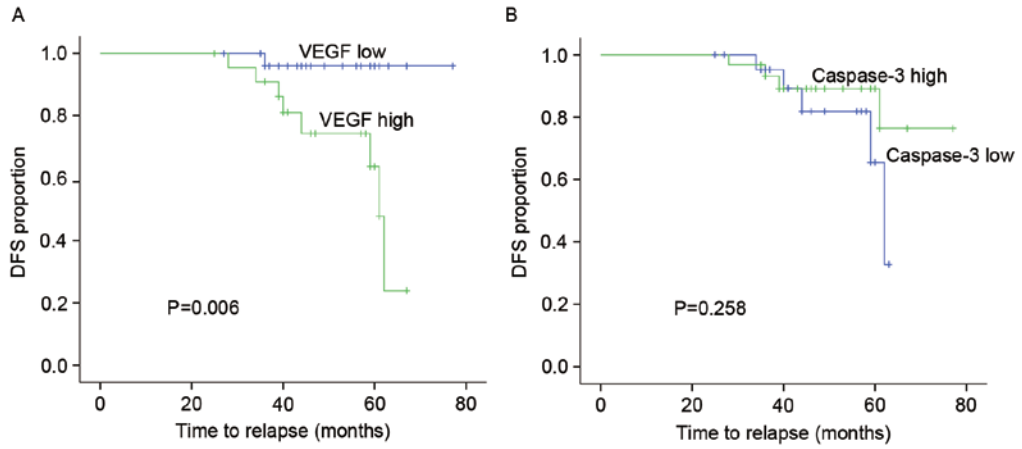

Figure 2. Kaplan-Meier survival analysis of DFS in MBC. (A) The high expression of VEGF was found to be significantly associated with a poor 5-year DFS rate in MBC (P<0.05). (B) The expression of caspase-3 exhibited no significant association with DFS in MBC. DFS, disease-free survival; VEGF, vascular endothelial growth factor; MBC, mucinous breast carcinoma.

percentage being 61.67% in the IDC-NOS group (P=0.042). Furthermore, 31 cases (57.4%) of MBC patients exhibited high caspase-3 expression (≥6), but only 20 cases (33.33%) in the IDC-NOS group exhibited high caspase-3 expression (P=0.028) (Table II).

Association between VEGF and caspase-3 expression and DFS in MBC patients. Since the expression, function and mechanism of IDC-NOS is already clear, the present study shows the associations between them in MBC. Kaplan-Meier log-rank test showed that the patients with high VEGF expression tended to experience shorter DFS times (P=0.006) compared with those with low expression in MBC. However, there was no association between caspase-3 expression and DFS time in MBC patients (Fig. 2).

Association between VEGF and caspase-3 expression and clinicopathological variables in MBC patients. There was a significant association between VEGF expression and age, nodal status and TNM stage in the MBC patients, but there was no significant association between caspase-3 expression and age, tumor stage, nodal status, TNM stage, estrogen

Table III. Association between VEGF, caspase-3 and other variables in MBC.

Variables	VEGF (-)	VEGF (+)	P-value	Caspase-3 (-)	Caspase-3 (+)	P-value
Age, years			0.013[a]			0.783
≤50	20	7		12	15	
>50	11	16		11	16	
Tumor stage			0.220			0.658
pT1	11	12		9	14	
pT2-3	20	11		14	17	
Nodal status			0.002[a]			0.717
Negative	30	15		20	25	
Positive	1	8		3	6	
TNM stage			0.008[a]			1.000
I-IIa	30	16		20	26	
IIb-IIIc	1	7		3	5	
ER			1.000			0.384
Negative	3	3		4	2	
Positive	28	20		19	29	
PR			0.107			0.554
Low[b]	12	14		10	16	
High[c]	19	9		13	15	
Ki-67, %			0.358			0.346
≤20	22	19		16	25	
>20	9	4		7	6	

[a]Significant difference between MBC and IDC-NOS (P<0.05); [b]PR <20%; [c]PR ≥20%. MBC, mucinous breast carcinoma; VEGF, vascular endothelial growth factor; ER, estrogen receptor; PR, progesterone receptor.

receptor (ER) status, progesterone receptor (PR) status or Ki-67 expression (Table III).

Cox analysis in MBC. Univariate Cox regression analyses showed higher VEGF score, positive nodal status and higher TNM stage were significant predictors of worse DFS. Caspase-3 expression had no significant predictive value in terms of DFS. Moreover, multivariate analysis analyzed the correlation between DFS and VEGF expression, nodal status and TNM stage, and found that TNM stage was significantly associated with worse DFS (Table IV). Ki-67 expression was also analyzed. The rate of Ki-67 (≤20%) expression was 75.93% in the MBC patients compared with 50% in the IDC-NOS patients (P<0.05), suggesting that MBC tumors may have lower proliferative ability than IDC-NOS tumors. However, Cox survival analysis showed that Ki-67 expression was not significantly associated with DFS in the MBC patients.

Discussion

The majority of previous MBC clinical studies found that MBC showed higher ER- and PR-positive rates, less lymph node metastasis, lower TNM stage and notably higher OS and DFS rates (16-20). The present study analyzed the basic information of MBC patients and compared it with that from IDC-NOS patients treated in the same period. The results were consistent with those of the previous studies, in which the patients with MBCs had a better prognosis than those with IDC-NOS.

Jao *et al* (21) studied 7 cases of MBC using electron microscopy and found that in addition to the abundant production of mucosubstance, MBC also featured the absence of myoepithelial differentiation and basal lamina deposition, the presence of notably developed cytoplasmic filamentous systems, a relatively scarcity of lysosomes, apparently and frequently well-developed intercellular junctions and a marked paucity of stromal vessels. These data suggest that the favorable clinical prognosis of MBC may be the result of multiple complicated factors (21).

Tumor growth requires constant vascular growth and remodeling so that solid tumors can exceed 1-2 mm^3 in size. VEGF and its receptors are key regulators of angiogenesis, meaning that they are attractive therapeutic targets (22). Microvessel density and tumor VEGF expression in hepatocellular carcinoma have previously been assessed, and the results indicated that upregulation of VEGF promoted angiogenesis, tumor growth and intrahepatic metastasis (23). VEGF-C-producing cancer cells may induce lymphatic vessel proliferation and dilation, resulting in cancer cell invasion into the lymphatic vessels and lymph node metastasis (24). Cancer treatment using a number of VEGF-targeted inhibitory agents is currently being assessed. VEGF-targeted therapy has been approved for the clinical treatment of metastatic triple-negative breast cancer (25). The paucity of stromal vessels in MBC may

Table IV. Cox univariate analysis and multivariate analysis of clinicopathological variables, including VEGF, for DFS in MBC.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
VEGF						
High	10.640	(1.326-85.403)	0.026[a]	5.881	(0.632-54.741)	0.120
Low						
Caspase-3						
High	0.473	(0.125-1.785)	0.269			
Low						
Age, years						
≤50	1.809	(0.452-7.244)	0.402			
>50						
Tumor stage						
pT2/pT3	1.941	(0.479-7.863)	0.353			
pT1						
Nodal status						
Positive	5.844	(1.452-23.529)	0.013[a]	0.477	(0.060-3.809)	0.485
Negative						
TNM						
II/III	11.689	(2.762-49.465)	0.001[a]	10.386	(1.230-87.703)	0.032[a]
I						
ER						
Positive	0.872	(0.172-4.409)	0.868			
Negative						
PR						
High[b]	0.542	(0.135-2.618)	0.386			
Low[c]						
Ki-67, %						
>20	1.299	(0.262-6.454)	0.749			
≤20						

[a]Significant difference between MBC and IDC-NOS (P<0.05). [b]PR ≥20%; [c]PR <20%. DFS, disease-free survival; MBC, mucinous breast carcinoma; VEGF, vascular endothelial growth factor; ER, estrogen receptor; PR, progesterone receptor; HR, hazard ratio; CI, confidence interval.

be an important adverse factor for angiogenesis (21). In the present study, VEGF expression was assessed in MBC and IDC-NOS samples, and VEGF expression in MBC was found to be lower than that in IDC-NOS. It was concluded that low VEGF expression in MBC may be associated with the paucity of tumor vessels and result in a better prognosis. In the MBC patients, high VEGF expression was significantly associated with primary lymph node metastasis and high TNM stage. Kaplan-Meier curves showed that VEGF was associated with DFS in the MBC patients, while in the Cox multivariate analysis model, only TNM stage was the independent prognostic factor.

A large amount of mucus is a typical feature of MBC samples. Norris and Taylor (8) found that the mucus in the tumor tissues plays an important role in the prognosis of the patients and the whole clinical course of the disease. Ahmed (10) noted that the formation of mucus is caused by the breakdown of cancer cells, followed by the degeneration of mitochondria, and that it leads to a marked decrease in tumor invasion. In addition to necrosis, apoptosis is a large part of cell disintegration. The caspases are a family of genes that are important for the maintenance of homeostasis via the regulation of cell death and inflammation (26). Caspase-3 is the key molecular factor in various apoptotic pathways (27,28). The present study examined caspase-3 expression in each group. MBC samples showed high caspase-3 expression, suggesting that caspase-3-mediated apoptosis may hinder tumor progression in MBC patients. Unexpectedly, caspase-3 expression was not associated with DFS or other clinicopathological parameters in the MBC patients.

The Ki-67 index has potential prognostic and predictive value in breast cancer, and has become an important, routinely used proliferation biomarker (29). The present study analyzed the expression of Ki-67 and found that the rate of Ki-67 (≤20%) expression in MBC was 75.93% compared with 50% in IDC-NOS. A significant difference exists between MBC and

IDC-NOS, which suggests that tumor cells of MBC may have a lower proliferation ability than those of IDC-NOS. However, Cox survival analysis showed that Ki-67 expression was not directly associated with DFS in MBC patients.

In conclusion, the present study revealed that high rate of hormone receptor and caspase-3, low expression of VEGF and Ki-67 and earlier TNM stage may contribute to improved prognosis of MBC compared with IDC-NOS. VEGF and caspase-3 may serve a role in mucus production, which is important in MBC progression. However, neither high expression of VEGF nor caspase-3 had a significant direct association with the DFS of patients with MBC. The mucinous breast cancer cell lines may need to be cultured in the future to explore the proliferation, invasion and migration ability of cancer cells and the exact role of mucus in tumor progression.

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