Expression of Malic Enzyme 3 in Breast Cancer and Precancerous Lesions: a Promising Novel Biomarker for Carcinogenesis and Prognosis

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Abstract

Purpose: While malic enzymes 1 (ME1) was correlated with breast cancer progression and prognosis, the association of ME3 (a homologue of ME1) with breast cancer is not known. The aim of this study is to explore the potential of ME3 as a biomarker in breast cancer carcinogenesis and prognosis.

Methods: A total of 107 patients confirmed with breast cancer were enrolled. The ME3 expression was evaluated by IHC and correlated with clinicopathological indicators.

Results: The ME3 positive immunostaining rate was higher in normal breast tissues and decreased stepwise from normal (97.60%) to usual ductal hyperplasia (91.1%), atypical ductal hyperplasia (64.2%), carcinoma in situ (62.5%) and invasive carcinoma (45.5%). Similarly, the decreasing tendency was observed for ME3 positive immunostaining rate from Tis (75.0%) through T1 (62.5%) and T2 (37.5%) to T3 (33.3%) and from stag 0 (75.0%) through I (72.0%), II (44.4%) to III (24.1%). ME3 expression was related with negative lymph node metastasis. Patients with positive expression of ME3 had better outcome. By incorporating ME3 into tumor TNM staging, the area under receiver operating characteristic curve for the 5-year survival was increased from 84.0% to 87.5%.

Conclusions: ME3 may be a promising biomarker for better prognosis for breast cancer patients.

Introduction

Breast cancer remains the leading cause of cancer-related death among females worldwide. It accounts for 24% of all cancer cases and 15% of all cancer deaths among females [1]. With dramatic changes in lifestyles and economy, the incidence and mortality rates of breast cancer in Chinese women have a horrendous growth, whereas the overall 5-year survival rate for Chinese women with breast cancer is much lower than American women (82% vs. 91%) [2–4]. Breast cancer has been a major public health problem in China [5,6].

It has been well known that changes in cell metabolism can contribute to transformation and tumor progression [7,8]. Malic enzymes (MEs), including three isoforms of ME1, 2 and 3, catalyze the oxidative decarboxylation of malate to pyruvate, with a concomitant reduction of $NAD(P)^+$ to NAD(P)H. The different isoforms of MEs have different cofactor specificity and sub-cellular localizations in mammals: the cytosolic $NADP^+$-dependent ME1, the mitochondrial $NAD(P)^+$-dependent ME2, and the mitochondrial $NADP^+$-dependent ME3 [9–11]. MEs are important for NADPH production: while ME1 is important for the NADPH production in the cytosol, ME3 takes a part in maintaining the redox homeostasis in mitochondria [12,13].

Recently, a growing number of evidence has indicated that ME family plays an important role in different types of cancer. Jiang et al reported that up-regulation of MEs induced p53 inhibition [13]. Lu and Murai et al demonstrated that ME1 inhibited proliferation and invasion by regulating NADPH homeostasis in

gastric cancer and colon cancer cell lines [14,15]. Ren et al showed that ME2 depletion increased reactive oxygen species (ROS) and NADP+/NADPH ratio, inhibited cell proliferation and induced cell death and differentiation in lung cancer cell [16]. Woo et al reported high-expression of ME2 was associated with poorer overall survival (OS) for patients with head and neck squamous cell carcinoma [17]. Dey et al found that ME3 depletion selectively killed ME2-null pancreatic cancer cells and Zhang Q et al discovered that ME3 promoted the proliferation, invasion and metastasis of pancreatic cancer cells [18,19]. Moreover, Liao et al reported that ME1 expression was positively correlated with large tumor size, high grade, poor survival and chemotherapy resistance, indicating that ME1 promoted breast cancer progression and was associated with poor prognosis [20].

In this study, we sought to characterize ME3 protein expression in breast precancerous and cancerous lesions, aiming to explore the potential of ME3 as a biomarker for carcinogenesis and prognosis in breast cancer.

Results

The clinicopathological characteristics of the patients

Table 1 summarized the clinicopathological characteristics of the 107 breast cancer patients. All the patients were confirmed with breast cancer, and were predominately at the invasive stage (92.5%), only 7.5% at CIS stage. Pathologically, there were 101 cases with ductal carcinoma, 3 cases with invasive lobular carcinoma, 1 case with medullary carcinoma, 1 case with mucinous carcinoma, and 1 case with mixed carcinoma. Based on the T staging, more than half of the patients were in T2 (59.8%), followed by T1 (29.9%), Tis (7.5%) and T3 (2.8%). About half of the patients (42.1%) had positive lymph node metastasis when they were diagnosed. 71.9% of the patients were in early stage based on TNM staging, including 7.5% patients in stage 0, 23.4% in stage I, 42.1% in stage II, and the rest (27.1%) in advanced stage III.

Table 1
Clinicopathological characteristics of 107 breast cancer patients.

Variables	No. of patients examined N (%)
Age	
< 50 years	64 (59.8)
≥ 50 years	43 (40.2)
Gender	
Male	0 (0)
Female	107 (100.0)
T staging	
Tis	8 (7.5)
T1	32 (29.9)
T2	64 (59.8)
T3	3 (2.8)
Lymph node metastasis	
No	62 (57.9)
Yes	45 (42.1)
Pathological staging	
0	8 (7.5)
I	25 (23.4)
II	45 (42.1)
III	29 (27.1)
Vascular cancer embolus	
No	87 (81.3)
Yes	20 (18.7)
ER status	
Negative	38 (35.5)
Positive	69 (64.5)

Variables	No. of patients examined N (%)
PR status	
Negative	42 (39.3)
Positive	65 (60.7)
Her-2 status	
Negative	45 (42.1)
Positive	39 (36.4)
Not available	23 (21.5)
Ki67 index	
Low expression (≤ 14%)	27 (25.2)
High expression (> 14%)	80 (74.8)

ME3 positive expression is decreased with the progression from precancerous to cancerous lesions

The immunohistochemical staining showed that the immunoreactivity for ME3 protein was located in the cytoplasm both in breast cancerous and precancerous tissue (Fig. 1). The ME3 positive expression is defined by semiquantitative scoring system as described in Materials and Methods. The ME3 positive immunostaining rate was higher in the normal breast tissue (97.60%, 82/84), and then the positive rate decreased from UHP (91.1%, 72/79) to AHP (64.2%, 43/67), CIS (62.5%, 40/64) and invasive carcinoma (45.5%, 45/99). The decreasing tendency was inversely correlated with the progression from precancerous to cancerous lesions (R^2 = 0.9414, P = 0.006, linear regression analysis, Table 2, Fig. 2).

Table 2
ME3 expression in the normal mammary tissue, precancerous and cancerous lesions.

Pathological classification	No. of lesions examined	Expression of ME3 protein		χ2	P
		Positive N (%)	Negative N (%)		
Normal	84	82 (97.6)	2 (2.4)	195.89	**0.000**
Usual hyperplasia	79	72 (91.1)	7 (8.9)		
Atypical hyperplasia	67	43 (64.2)	24 (35.8)		
Carcinoma in situ	64	40 (62.5)	24 (37.5)		
Invasive carcinoma	99	45 (45.5)	54 (54.5)		

ME3 is associated with better prognosis in breast cancer patients

The median follow-up of the entire cohort was 73.7 months (range 11.7–94.8 months) and 21 (19.6%) patients died because of the deteriorated breast cancer during the follow-up. The survival rates of 1-, 3- and 5-year were 98.1%, 87.9%, and 82.2%, respectively. It was noteworthy that the median survival time of the 21 dead patients was only 36.6 months, ranging from 11.7 to 67.2 months. Of the 21 died cases, one third (33.3%) was in early stage I and II, and the rest (66.7%) was in advanced stage III. In this study, the younger patients with ages ≤ 40 years accounted 16.8% (18/107) and about half of the patients (44.4%, 8/18) were in advanced stage III.

The Kaplan–Meier survival analysis showed that the patients with positive expression of ME3 had significant better outcome than those with negative expression ($\chi2 = 8.233$, $P = 0.004$, Fig. 4).

In this study, lymph node metastasis ($\chi2 = 12.284$, $P = 0.000$), TNM staging progression ($\chi2 = 18.232$, $P = 0.000$) and high expression of Ki67 index ($\chi2 = 5.155$, $P = 0.024$) were also negatively associated with survival (Supplementary Table S1). However, no influence was observed for age, T staging, vascular cancer embolus, ER status, PR status and Her-2 status on survival (Supplementary Table S1).

ME3 is inversely associated with the progression of breast cancer

Furthermore, linear regression analysis showed that ME3 expression decreased from Tis (75.0%, 6/8) to T1(62.5%, 20/32), T2 (37.5%, 24/64) and T3 (33.3%, 1/3), ($R^2 = 0.9394$, $P = 0.031$, Table 3, Fig. 3A). The analysis on TNM staging and ME3 expression showed ME3 positive immunostaining rate was much higher in stage 0 (75.0%, 6/8) and stage I (72.0%, 18/25) than in stage II (44.4%, 20/45) and stage III (24.1%, 7/29) ($\chi2 = 14.953$, $P = 0.002$, Table 3). Similarly, the decreasing tendency was also significant from stage 0 to I, II and III ($R^2 = 0.9283$, $P = 0.037$, linear regression analysis, Fig. 3B). ME3 positive expression in breast cancer tissue was related with negative lymph node metastasis ($\chi2 = 6.393$, $P = 0.011$). No significant difference was observed for ME3 expression with age, vascular cancer embolus, ER status, PR status, Her-2 status, or Ki67 index (Table 3)

Table 3
Relationship between ME3 expression and clinicopathological characteristics in breast cancer.

Variables	No. of patients examined	Expression of ME3 protein		χ2	P
		Positive(n = 51) N (%)	Negative(n = 56) N (%)		
Age				1.914	0.166
< 50 years	64	27 (42.2)	37 (57.8)		
≥ 50 years	43	24 (55.8)	19 (44.2)		
T staging				8.117	**0.044**
Tis	8	6 (75.0%)	2 (25.0%)		
T1	32	20 (62.5%)	12 (37.5%)		
T2	64	24 (37.5%)	40 (62.5%)		
T3	3	1 (33.3%)	2 (66.7%)		
Lymph node metastasis				6.393	**0.011**
No	62	36 (58.1)	26 (41.9)		
Yes	45	15 (33.3)	30 (66.7)		
Pathological staging				14.953	**0.002**
0	8	6 (75.0)	2 (25.0)		
I	25	18 (72.0)	7 (28.0)		
II	45	20 (44.4)	25 (55.6)		
III	29	7 (24.1)	22 (75.9)		
Vascular cancer embolus				0.070	0.791
No	87	42 (48.3)	45 (51.7)		
Yes	20	9 (45.0)	11 (55.0)		
ER status				0.730	0.393
Negative	38	16 (42.1)	22 (57.9)		
Positive	69	35 (50.7)	34 (49.3)		
PR status				2.537	0.111
Negative	42	16 (38.1)	26 (61.9)		
Positive	65	35 (53.8)	30 (46.2)		

Variables	No. of patients examined	Expression of ME3 protein		χ2	P
		Positive(n = 51) N (%)	Negative(n = 56) N (%)		
Her-2 status				1.439	0.487
Negative	45	22 (48.9)	23 (51.1)		
Positive	39	16 (41.0)	23 (59.0)		
Not available	23	13 (56.5)	10 (43.5)		
Ki67 index				3.389	0.066
Low expression (≤ 14%)	27	17 (63.0)	10 (37.0)		
High expression (> 14%)	80	34 (42.5)	46 (57.5)		

Receiver operating characteristic curve

Using multivariate logistic regression and receiver operating characteristic curve (ROC), we explored the potential of ME3 expression in the classification of 5-year survival outcome. Logistics regression analysis showed that TNM staging was the influencing factor for 5-year survival outcome in the available clinical information in this study. Using TNM alone, the 5-year survival model based on the prediction probability showed that the area under ROC curve was 84.0% (Fig. 5A). Using TNM combined with ME3 expression, the area under ROC curve increased to 87.5% (Fig. 5B), suggesting a potential role of the ME3 in prediction of patients at risk of breast cancer death.

Discussion

To the best of our knowledge, this is the first report to investigate the potential of ME3 as a biomarker in breast cancer carcinogenesis and progression. The present study demonstrated a stepwise decrement for ME3 protein expression at the different stages of breast malignant progression, indicating that ME3 aberrant loss of expression may be involved in breast carcinogenesis.

Furthermore, ME3 positive immunostaining rate was decreasing from Tis through T1, T2 to T3 and from stag 0 through I, II to III. In addition, ME3 expression was strongly related with lymph node status. The close association between ME3 expression and pathological staging suggests that ME3 may involve in the progression of in-situ invasion and lymph node metastasis of breast cancer. Meanwhile, patients with ME3 positive expression have a better survival than the negative expression of ME3.

Breast cancer is the most frequent cancer among female and TNM staging at diagnosis is still one of the most important factors affecting prognosis so far. Breast cancer has a heterogeneous prognosis of the 5-year survival rate varies substantially among patients, range from 27% for distant metastasis to 86% for

regional breast cancer and 99% for localized breast cancer in the report from the American Cancer Society [21]. In this study, addition of ME3 expression into TNM staging has a stronger predictive value of 5-year survival outcome than TNM staging alone. Hence, ME3 could be a potential biomarker for better outcome of breast cancer patients.

It has not been well characterized for the ME3 and the associated metabolic pathway in carcinogenesis, especially in breast cancer. The mechanism of ME3 aberrant expression in breast cancer is largely unknown. Biologically, ME3 catalyzes a reaction by oxidizing malate to pyruvate and concomitantly reducing $NADP^+$ to NADPH and this reaction is crucial for maintaining the NADPH pool in mitochondria [22,23]. As NAPDH is the essential molecule for antagonizing mitochondrial ROS (mROS), ME3 downregulation may enhance the accumulation of mROS. It has been well recognized that mROS overproduction would promote carcinogenesis and tumor progression by inducing genetic instability, modifying gene expressions, and activating diverse signaling [24,25]. Therefore, ME3 downregulation may take a part in breast carcinogenesis and breast cancer progression via perturbing the homeostasis of $NADPH/NADP^+$ in mitochondria, leading to accumulation of mROS. Further investigation is required to validate the hypothesis.

It is interesting to note that ME3 and ME1 showed opposite effect on diagnosis on the survival of breast cancer patients. While it has been extensively studied that higher ME1 expression is associated with worse prognosis of breast cancer patients than the lower ME1 expression [26], the higher ME3 expression was associated with a better prognosis of breast cancer patients than the lower ME3 expression. Because ME1 and ME3 share the same catalytic function catalyzing the oxidative decarboxylation of malate, the different effect of the enzymes on prognosis may emanate from their different locations. ME1 is located in cytoplasm, whereas ME3 is located in mitochondria. Because it is well recognized that the vicious cycle of ROS in mitochondria is important for carcinogenesis and cancer progression [27–29], it is conceivable that this vicious cycle is to some extent associated with ME3 activity. The lower ME3 expression may enhance the vicious cycle and hence may be correlated with the progression from normal to precancerous tissue and to cancerous tissue as well as the progression from early to later stage of breast cancer.

In conclusion, our data supported ME3 as a potential biomarker for breast carcinogenesis as well as for the progression of breast cancer patients. ME3 positive expression may be a promising biomarker for better outcome for breast cancer patients.

Materials And Methods

Patients

107 breast cancer patients were retrieved in this study from a database, which has been established by The State Key Laboratory of Esophageal Cancer Prevention & Treatment and Henan Key Laboratory of Esophageal Cancer Research in The First Affiliated Hospital of Zhengzhou University. All the patients had

been underwent mastectomy from August 2012 to December 2014 without any radiation therapy or chemotherapy prior to surgery before operation. All patients were females with a mean age of 48.41±11.18 years and a median age of 46 years (range from 26 to 82 years). Staging for breast cancer were based on American Joint Committee on Cancer (AJCC) TNM staging system in 2017 [30]. The follow-up was performed with hospital medical records, either through telephone or through home interview to each patient yearly until death. The last follow-up was May 2020. Overall survival time was calculated from the day of mastectomy to death or to the last follow-up date. The study protocol was approved by the Medical Ethics Committee of Zhengzhou University.

Tissue collection and processing

A total of 107 breast cancer tissues and 87 matched adjacent non-cancerous tissues were collected from surgically resected specimens from the patients with primary breast cancer. The tissues were routinely 10% neutral formalin fixed, paraffin-embedded and then sectioned. Serial 4 μm sections were prepared for histopathological analysis (hematoxylin and eosin stain) and immunohistochemical staining.

Histopathological analysis

Histopathological diagnoses and staging for breast cancer and adjacent non-cancerous lesions were made based on WHO criteria in 2012 [31]. The epithelial proliferative lesions in mammary gland acini and duct were categorized as “normal”, usual hyperplasia (UHP), atypical hyperplasia (AHP) and carcinoma in situ (CIS). Histopathologically, UHP was usually found adjacent to the tumor tissue; AHP and CIS were frequently observed as isolated lesions in the surgically resected breast specimens. Pathologically, of the 87 cases with adjacent cancer tissue, there were 84 cases identified with normal tissue (96.6%, 84/87), 79 with UHP (90.8%, 79/87), 67 with AHP (77.0%, 67/87) and 64 with CIS (73.6%, 64/87). Histological examination was carried out by two pathologists independently.

Immunohistochemical staining

Follow the manufacturer’s instructions, tissue sections were deparaffinized, rehydrated, subjected to antigen retrieval. Polyclonal rabbit anti-human ME3 antibody was purchased from Sigma (HPA038473, USA). The samples were incubated with primary antibody (ME3, 1:400) overnight at 4 °C. The secondary antibody was conjugated to horseradish peroxidase. The immunoreactivty for ME3 was visualized through adding diaminobenzidine and subsequently counterstained with hematoxylin. The sections were evaluated under microscope by two pathologists independently who were blinded to the patients’ clinical information.

According to immunohistochemical scoring system previously with some modification [32,33], ME3 immunostaining score (S) was determined semiquantitatively by multiplying the percentage of positive cells (P) with intensity (I), according to the formula: S= P×I. The range score for percentage of positive cells is 0 to 4 (0, 1%; 1, 1%-10%; 2, 11%-50%; 3, 51%-75%; 4, 76%-100%). The range score for intensity is 0 to 3 (0, no staining; 1, weak; 2, moderate; 3, strong.). The index score was obtained a range from 0 to 12.

The final immunostaining results were denoted as negative expression of ME3 (0–1 scores) and positive expression of ME3 (≥2 scores).

Statistical analysis

All statistical analyses were conducted using IBM SPSS version 21.0. Data was represented as the mean ± standard deviation for continuous variables and frequency (number-percent) for categorical data. Relationships of ME3 expression and clinicopathological features were evaluated by the chi-square ($\chi 2$-value) and linear regression analysis (R^2-value). The Kaplan–Meier survival analysis was used to estimate the association between eligible variables and survival. Cox proportional hazards regression models were used for multivariate survival analysis in stepwise regression manner. Receiver operating characteristic curve was based on the prediction probability of 5-year survival outcome from the logistic regression model, and the area under ROC curve was used to assess the classification performance of the model. Any result with a P-value of less than 0.05 was considered as statistically significant.

Declarations

Acknowledgments

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Authors' contributions

Conception and design of the study: XH and DY; Sample acquisition and analysis: DD, and DY; Histopathological diagnosis: XML and DD; Manuscript preparation: All authors; Approval of the final version: All authors.

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

Informed consent was obtained from patients in this study. The human study protocol was in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans were approved by the Medical Ethics Committee of Zhengzhou University.

Consent to participate

Not applicable.

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Figures

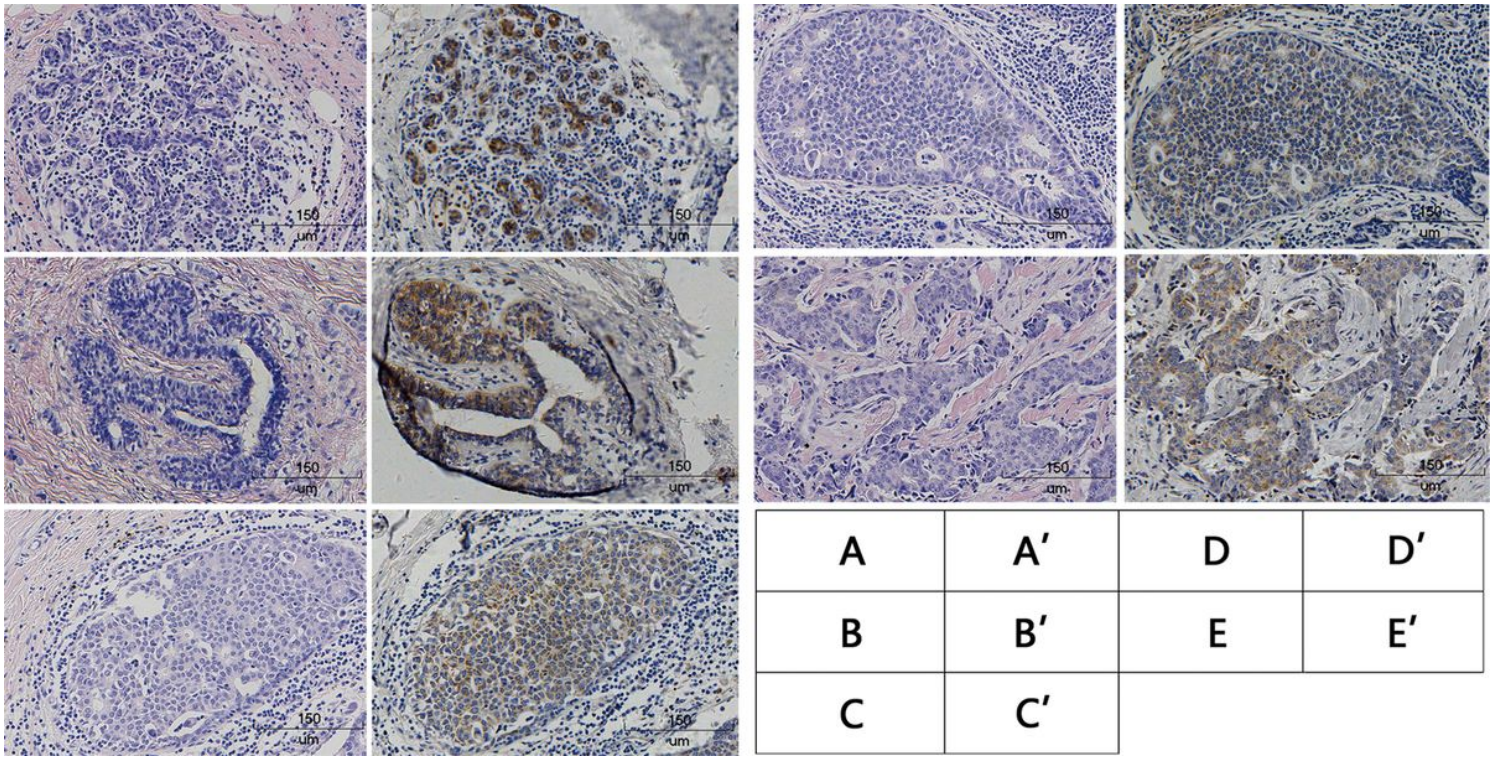

Figure 1

A-E are the microscopic representatives of hematoxylin and eosin staining for normal tissue (A), usual hyperplasia (B), atypical hyperplasia (C), carcinoma in situ (D) and invasive carcinoma (E). A′–E′ are the matched tissues of ME3 immunostaining. Scale bar = 150 μm.

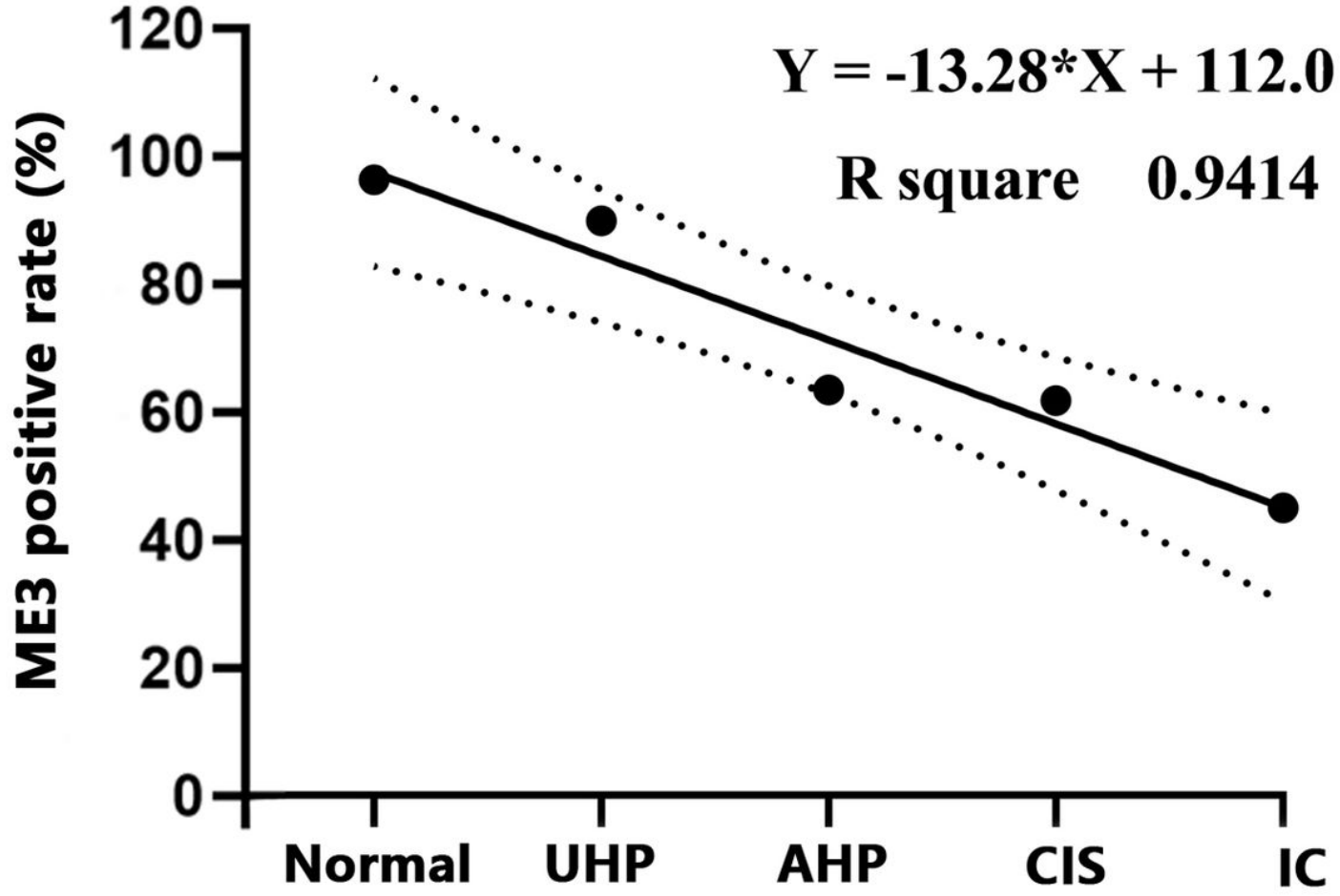

Figure 2

The ME3 positive immunostaining rate decreased from normal to usual hyperplasia (UHP), atypical hyperplasia (AHP), carcinoma in situ (CIS) and invasive carcinoma (IC) (R2=0.9414, P=0.006, linear regression analysis). Data are from table 2.

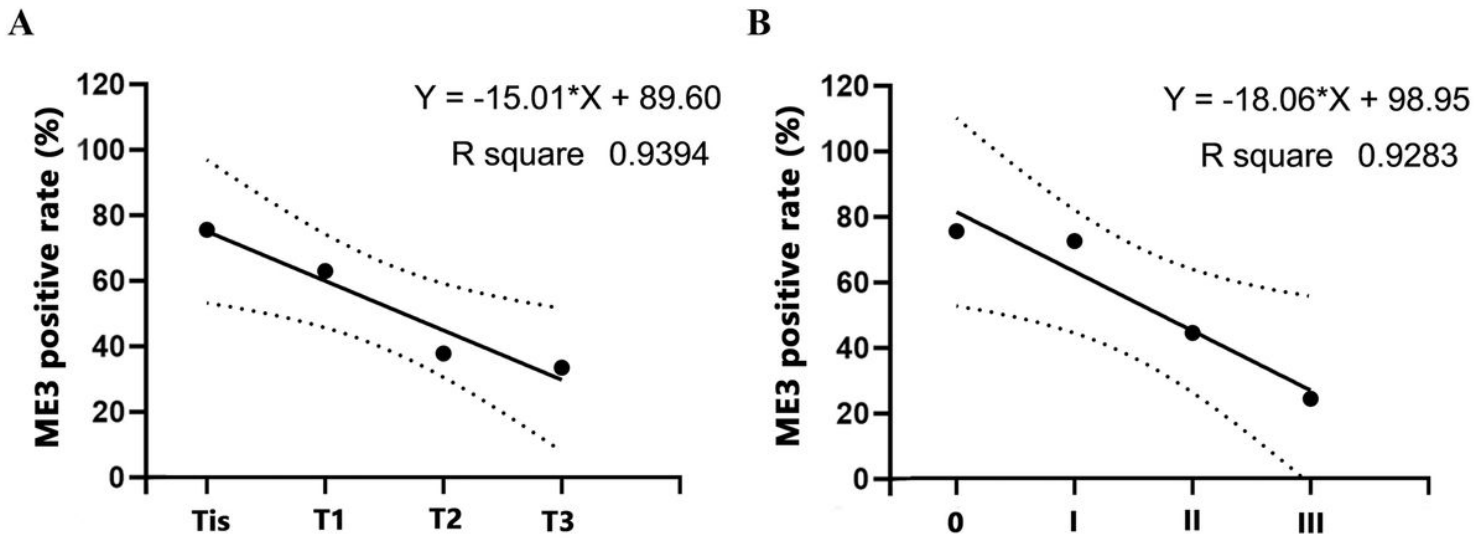

Figure 3

Linear regression of ME3 positive immunostaining rate in T staging and TNM staging. (A) ME3 positive immunostaining rate in Tis, T1, T2 and T3, (R2=0.9394, P=0.031); (B) ME3 positive immunostaining rate in stage 0 to I, II and III (R2=0.9283, P=0.037). Data are from table 3.

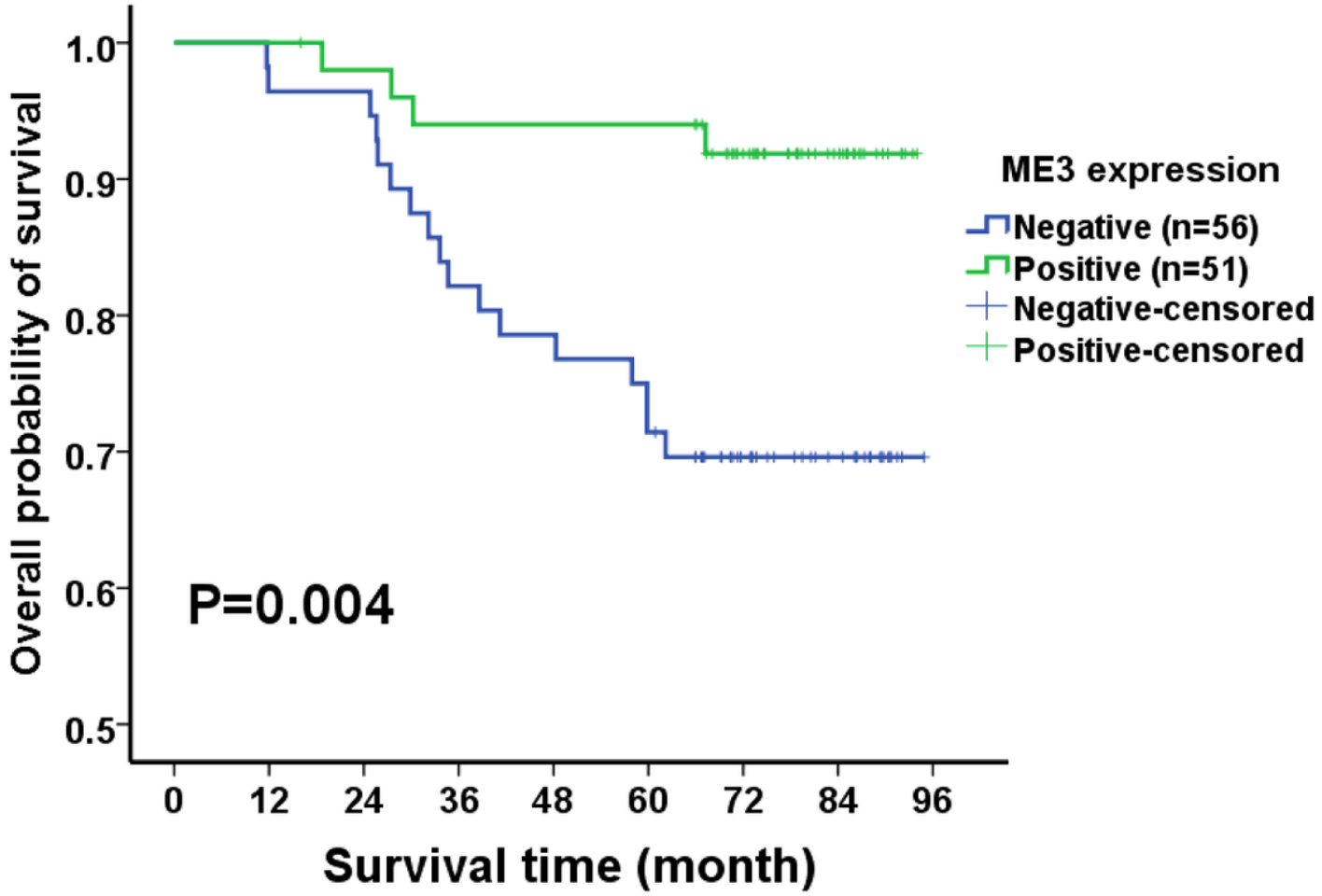

Figure 4

The Kaplan–Meier survival analysis showed that the patients with positive expression of ME3 had significant better outcome than those with negative expression ($\chi 2=8.233$, P=0.004).

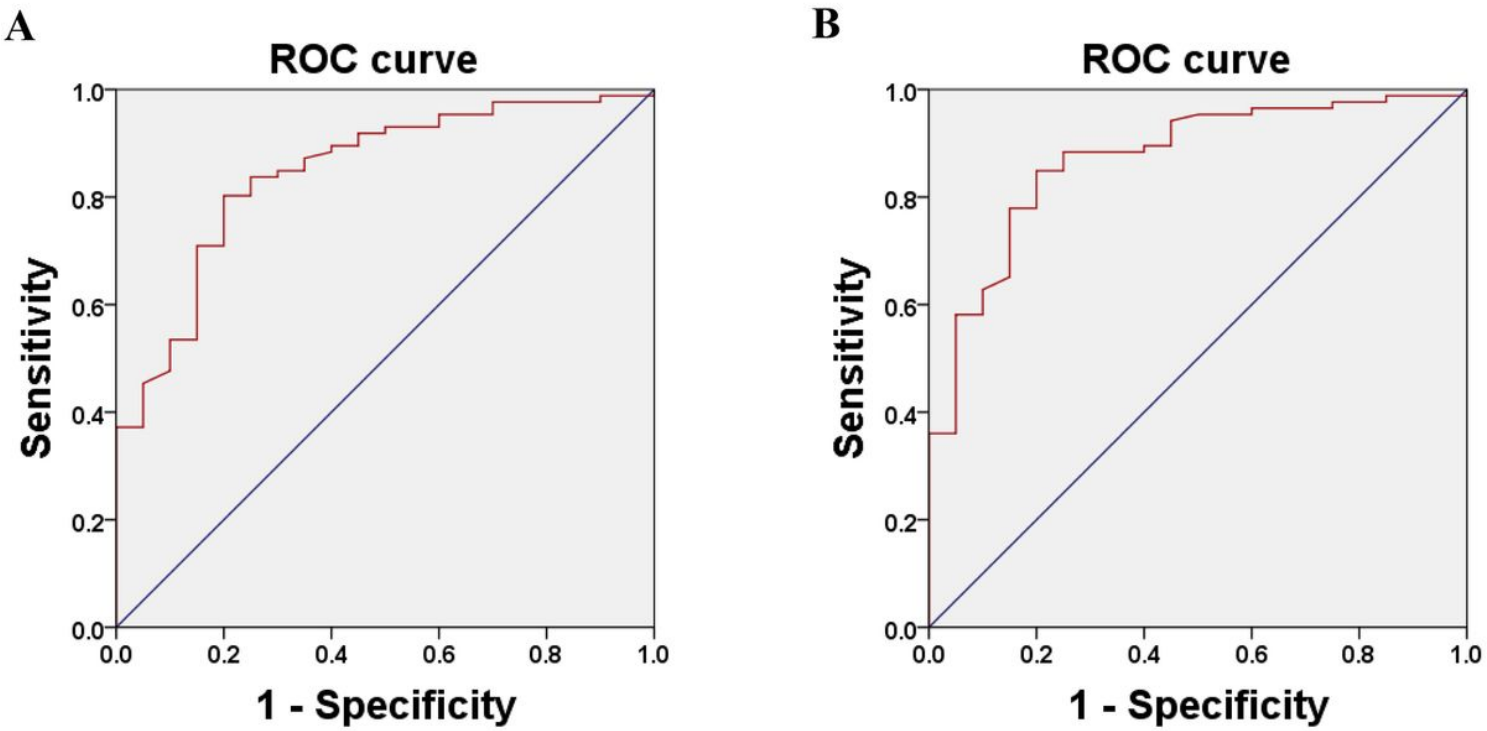

Figure 5

Receiver operating characteristic curves for prediction of breast cancer 5-year survival outcome. (A) 5-year overall survival rate based on tumor TNM staging (the area under ROC curve =84.0%); (B) 5-year overall survival rate based on tumor TNM staging combined with ME3 expression (the area under ROC curve =87.5%).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.docx