Pathology - Research and Practice 215 (2019) 490–498

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

MFG-E8 overexpression is associated with poor prognosis in breast cancer patients

Lifeng Yu[a,b], Lin Zhao[a,b], Zhen Jia[a,b], Jia Bi[a,b], Qian Wei[a,b], Xinyue Song[a,b], Longyang Jiang[a,b], Shu Lin[a,b], Minjie Wei[a,b,*]

[a] Department of Pharmacology, School of Pharmacy, China Medical University, Shenyang, Liaoning, 110122, China
[b] Liaoning Key Laboratory of molecular targeted anti-tumor drug development and evaluation China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang City, 110122, Liaoning, China

ARTICLE INFO

Keywords:
MFG-E8
Breast cancer
Clinicopathological characteristics
Prognosis

ABSTRACT

Background: MFG-E8(Milk fat globule-EGF factor 8), a secreted glycoprotein, plays an exceptional role in various diseases. MFG-E8 overexpression is found in a variety of cancers. However, it remains unclear whether MFG-E8 overexpression is associated with the clinicopathological characteristics and prognosis of human breast cancer.
Materials and methods: In this study, we detected the expression and localization of MFG-E8 protein in breast cancer and cancer-adjacent tissues using immunohistochemical staining, Western blot analysis and immunofluorescence. We analyzed the association between MFG-E8 expression and clinical characteristics and outcomes of breast cancer patients with different HR and HER2 statuses.
Results: Our results confirmed that MFG-E8 expression increased significantly in breast cancer compared with cancer-adjacent tissues by immunohistochemical staining ($P < 0.001$). Similarly, the Western blot results further confirmed the increased expression of MFG-E8 in breast cancer compared with cancer-adjacent tissues ($P = 0.001$). Immunofluorescence staining showed that MFG-E8 was mainly localized in the cytoplasm and membrane of tumor cells, consistent with the immunohistochemical staining results. The high expression levels of MFG-E8 showed a greater association with lymph node metastasis, TNM stage and histological grade ($P < 0.001$). Moreover, high MFG-E8 expression was related to a shortened overall survival (OS) ($P < 0.001$) and disease-free survival (DFS) ($P < 0.001$). Bioinformatics analysis with a Kaplan-Meier plotter also demonstrated a strong association of *MFG-E8* mRNA overexpression with a short OS and DFS compared with low MFG-E8 expression ($P = 0.040$, $P = 0.005$).
Conclusions: Our findings indicate that MFG-E8 may be a potential marker for poor prognosis and survival in breast cancer.

1. Introduction

Breast cancer in females is the most common malignant cancer worldwide and remains a leading cause of cancer-related death [1–3]. Clinically, there are different histological forms of breast cancer, which may result in diverse therapy protocols and prognoses [4–7]. Until now, some biomarkers have been available for guiding pharmacy and predicting prognosis in breast cancer. Estrogen receptor (ER) and progesterone receptor (PR) are likely to respond to endocrine therapy [8–10]. Patients with high HER2 expression can use anti-HER2 (human epidermal growth factor receptor-2) therapy (trastuzumab) [11–13]. Clinically, Ki67 is used to determine prognosis, although methodological problems persist [14,15]. Hence, the search for new biomarkers for breast cancer is crucial.

MFG-E8, also known as lactadherin, is a secreted glycoprotein in humans that is encoded by the *MFG-E8* gene [16,17]. MFG-E8 is composed of two-repeated EGF-like domains, two repeated discoidin-like domains (C domains), and a mucin-like domain. In the EGF-like domain, there is an integrin-binding motif that contributes αvβ3 and αvβ5 integrins heterodimers for cell adhesion and causes integrin-mediated signal transduction [17,18]. This glycoprotein is located and expressed widely in humans and has multiple functions. Traditionally, MFG-E8

Abbreviations: OS, overallsurvival; DFS, disease-free survival; MFG-E8, milk fat globule-EGF factor 8; ER, estrogen receptor; PR, progesterone receptor; HR, hormone receptor; HER2, human epidermal growth factor receptor-2

* Corresponding author at: Department of Pharmacology, School of Pharmacy, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang City, 110122, Liaoning, China.
E-mail address: weiminjiecmu@163.com (M. Wei).

https://doi.org/10.1016/j.prp.2018.12.036
Received 21 September 2018; Received in revised form 10 December 2018; Accepted 30 December 2018

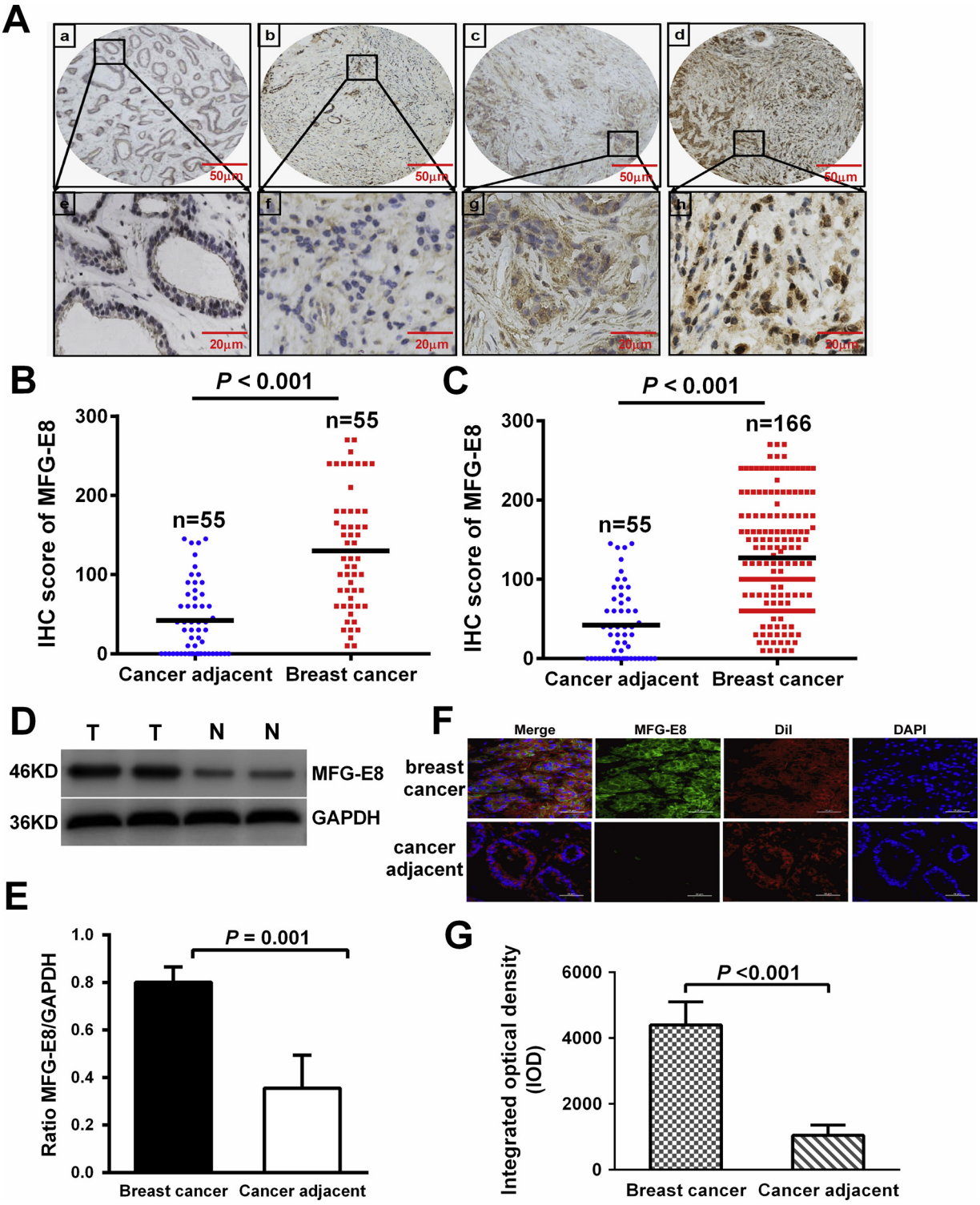

Fig. 1. Overexpression and location of MFG-E8 in breast cancer tissues. (A) Representative immunohistochemical staining of MFG-E8 in cancer-adjacent tissues (Aa, Ae) and breast cancer tissues (Ab-d, f–h). b and f, c and g, d and h show weak, moderate and strong staining, respectively; 50 μm for 200× and 20 μm for 400 × . (B) and (C) Statistics for the immunohistochemical staining of MFG-E8 in cancer-adjacent tissues and breast cancer tissues; (B) compared with 55 pairs of samples of cancer-adjacent and breast cancer by Wilcoxon signed-rank test; (C) compared with 55 cases of cancer-adjacent tissues and 166 cases of breast cancer tissues; (D) Six pair of fresh breast cancer tissues (T) and cancer-adjacent tissues (N) were selected and analyzed for MFG-E8 protein expression by Western blotting. GAPDH was used as a loading control. (E) Statistics for the gray scanning scales of MFG-E8/GAPDH in breast cancer tissues and cancer-adjacent tissues. (F) Localization and expression of MFG-E8 in human breast cancer and cancer-adjacent tissues using immunofluorescence staining. Green color from FITC represents MFG-E8 expression. Blue color from DAPI represents cell nuclei. Red color from DiI represents cell membrane. Scale bar: 50 μm for 200 × . (G) The integrated optical density (IOD) was analyzed using NIS Elements software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

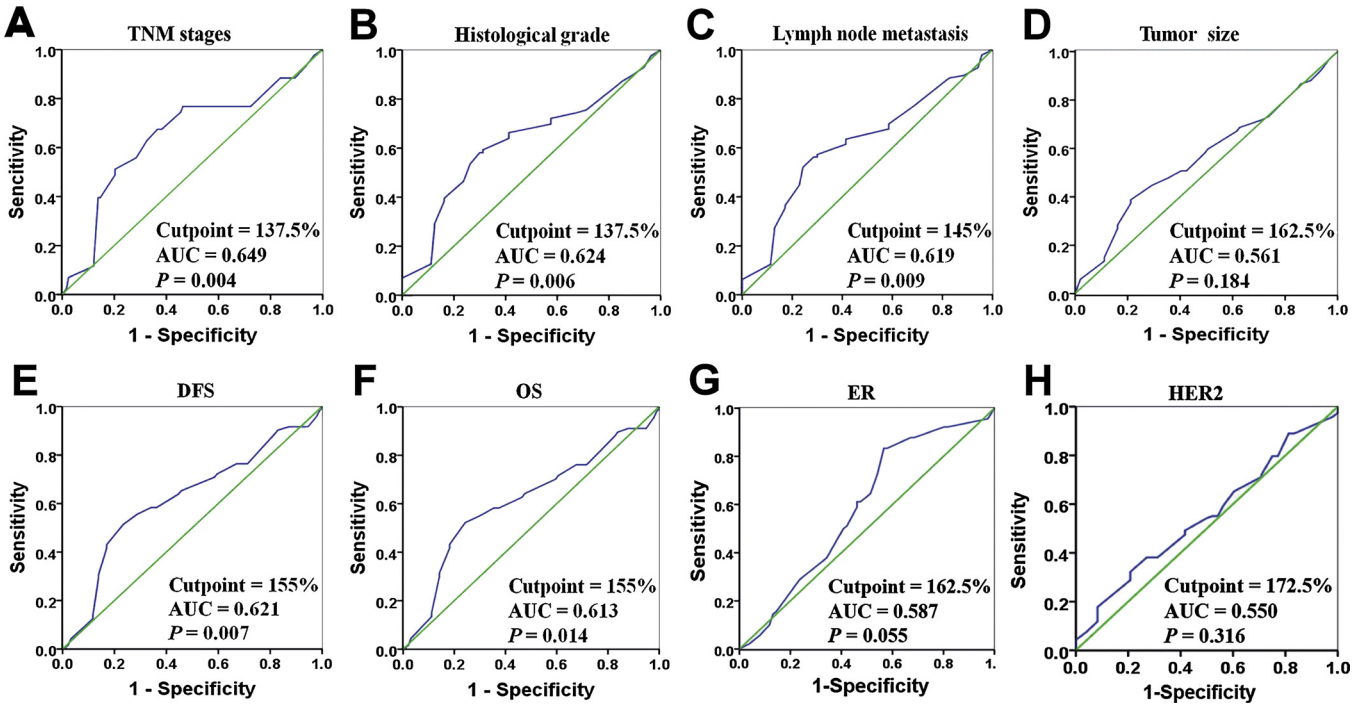

Fig. 2. Receiver operating characteristic curves were used to determine the cutpoint score for MFG-E8 overexpression in breast cancer. The ROC curves displayed a discrimination of the expression levels of MFG-E8 by TNM stage, histological grade, lymph node metastasis, tumor size, DFS, OS, ER and HER2 status. The specificity and sensitivity for each outcome were plotted, and the areas under the curve (AUC) and *P* value are indicated. The AUC for TNM stage had the biggest area and the *P* value was the smallest of all. (A) TNM stage; (B) histological grade; (C) lymph node metastasis; (D) tumor size; (E) DFS; (F) OS; (G)ER status.; (H) HER2 status.

has been verified as an important factor for the clearance of apoptotic cells and a prominent factor in the immune system [19–25]. In recent years, some studies have demonstrated that MFG-E8 is a crucial factor in various diseases, including sepsis, age-related diseases and tumors [26–29].

Previous experiments have shown that MFG-E8 is significantly upregulated in carcinomas compared with normal tissues such as colorectal cancers, oral squamous cell carcinomas, and hepatocellular carcinomas [26]. However, Chuanwei Yang et al have demonstrated that the expression of *MFG-E8* mRNA is upregulated in triple-negative breast cancer patients and cell lines [30]. To date, it remains unclear whether the expression of MFG-E8 is associated with HR and HER2 status and the related clinical outcomes in breast cancer patients

In this study, we investigated the expression of MFG-E8 protein in the cancerous and cancer-adjacent tissues of breast cancer patients by immunohistochemistry and Western blotting. We found that overexpression of MFG-E8 was associated with a poor prognosis in breast cancer patients. These results suggest that MFG-E8 might be a potential biomarker for predicting prognosis in breast cancer.

2. Materials and methods

2.1. Patients and tissue samples

This study was approved by the Medical Ethics Committee of China Medical University. Due to the retrospective nature of the study, the Ethics Committee waived the need for written informed consent by the patients.

Human breast cancer tissue samples were obtained from 166 female patients who underwent surgery at the First Affiliated Hospital of China Medical University from 2008 to 2012. Fifty-five cancer-adjacent tissues were also collected as a control. The diagnosis of breast cancer and cancer-adjacent samples were confirmed by pathological staining.

The average age of the breast cancer patients was 51 years (range, 28–78 years). Of the 166 breast cancer patients, 8 had intraductal carcinoma, 129 had infiltrating ductal carcinoma, 6 had infiltrating lobular carcinoma, and 23 had other types of carcinomas including medullary carcinoma, mucinous carcinoma, and cribriform carcinoma. The histological grade of the breast cancer was determined in accordance with the World Health Organization grading system. The stage of the breast cancer was evaluated in 166 patients according to the TNM staging system as follows: stage I (n = 22), stage II (n = 101), and stage III˙IV (n = 43). The clinicopathological data for the patients, including age, menopausal status, tumor size, lymph node metastasis, ER, PR, HR and HER2 status, were retrospectively retrieved from the medical records.

2.2. Tissue microarray (TMA) and immunohistochemistry

Paraffin blocks, including representative breast cancer samples and cancer-adjacent samples, were selected by reviewing all the hematoxylin and eosin-stained slides. Tissue cores with a diameter of 1.5 mm were extracted from each paraffin block and precisely arrayed into a new paraffin recipient block with a maximum of 200 cores using the Organization Microarrayer (Pathology Devices, USA). Sections with a thickness of 4 μm were obtained from formalin-fixed and paraffin-embedded TMA blocks, mounted on poly-L-lysine-coated glass slides, and used for immunohistochemistry.

Sections of the tissue microarray were deparaffinized with xylene, rehydrated in a graded alcohol series, and then washed in distilled water. Subsequently, they were heated in a microwave oven with 10 mM sodium citrate buffer (pH 6.0) for 10 min to retrieve antigen. Sections were incubated with 3% hydrogen peroxide at 37 ℃ for 30 min to block endogenous peroxidase activity. The nonspecific protein binding sites were blocked with 10% normal goat serum at 37 ℃ for 30 min. Then, the sections were incubated with mouse anti-MFG-E8 antibody (1:50 dilution; Santa Cruz) overnight at 4 ℃, followed by incubation with biotin conjugated secondary antibody (1:200 dilution; LSAB kit; Dako, Glostrup, Denmark) for 30 min at 37 ℃. The slides were incubated in streptavidin horseradish peroxidase for an additional

Table 1
Association of MFG-E8 expression with clinicopathological characteristics of breast cancer.

	MFG-E8 expression, n (%)		
	High	Low	*P*–value[a]
Age at diagnosis			
≤ 51 (year)	35(44.9)	43 (55.1)	0.827
> 51 (year)	38 (43.2)	50 (56.8)	
Menopausal status			
Pre-Menopause	31 (44.8)	36 (55.2)	0.864
Post-Menopause	43 (43.4)	55 (56.6)	
Tumor size (cm)			
≤3.0	39 (39.4)	60 (60.6)	0.148
> 3.0	34 (50.7)	33 (49.3)	
Nodes metastasis			
0	19 (27.5)	50 (72.5)	< **0.001**
1~3	26 (47.3)	29 (52.7)	
4~9	16 (76.2)	5 (23.8)	
≥10	12 (57.1)	9 (42.9)	
TNM stage			
I	5 (22.7)	17 (77.3)	< **0.001**
II	39 (38.6)	62 (61.4)	
III~IV	29 (67.4)	14 (32.6)	
Histological grade			
G1	28 (66.7)	14 (33.3)	< **0.001**
G2	22 (50.0)	22 (50.0)	
G3	23 (28.7)	57 (71.3)	
Histological type			
Intraductal carcinoma	2 (27.5)	6 (72.5)	0.497
Infiltrating ductal carcinoma	60 (46.5)	69 (53.5)	
Infiltrating lobular carcinoma	3 (50.0)	3 (50.0)	
Others	8 (34.8)	15 (65.2)	
ER status			
Negative	31 (50.8)	30 (49.2)	0.176
Positive	42 (40.0)	63 (60.0)	
PR status			
Negative	32 (41.6)	45 (58.4)	0.559
Positive	41 (46.1)	48 (53.9)	
HR status			
Negative	19 (48.7)	20 (51.3)	0.552
Positive	55 (43.3)	72 (56.7)	
HER-2 status			
Negative	32 (52.5)	29 (47.5)	**0.093**
Positive	41 (39.0)	64 (61.0)	

[a] *P*-value obtained from Pearson Chi-Square test or Fisher's Exact Test. HR + was defined as either ER + or PR+; HR- was defined as both ER- and PR-.

30 min, washed three times with PBS, and stained with DAB (3,3-diaminobenzidine) for approximately 2 min. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. Sections in PBS instead of anti-MFG-E8 primary antibody served as a negative control.

2.3. Evaluation of immunohistochemistry

The immunostaining was assessed by two pathologists independently who were blinded to the experimental conditions. The staining intensity was scored as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percentage of stained tumor cells was calculated as 0–100%. Five sections were used from each sample, and five fields were randomly selected in each section. The final immunoreactivity score was determined by the intensity score by multiplying by the percentage of positively stained cells. Scores was assigned using 5% increments (0%, 5%, 10%, ….300%) as previously reported [31,32]. The average score of each sample was used to assess the cutoff point for high or low MFG-E8 expression using receiver operating characteristic (ROC) curves. The sensitivity and specificity for each outcome of the breast cancer patients under this study was plotted.

2.4. Western blot analysis

Western blot analysis was performed according to a previous study [33]. Briefly, total protein from 6 pairs of fresh tumor and cancer-adjacent samples were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% fat free milk/TBST solution and incubated with primary MFG-E8 antibody (Santa Cruz, USA) at 1:100 overnight at 4 °C. After washing with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at 37 °C. The proteins of MFG-E8 were visualized using ECL development solution, and GAPDH was used as a loading control.

2.5. Immunofluorescence

The tissues of 6 pairs of fresh tumors and cancer-adjacent samples were cut into 8 mm sections. The sections were then fixed in 4% paraformaldehyde dissolved in PBS for 20 min. After blocking with 10% normal goat serum at 37 ℃ for 30 min, the sections were incubated with 1:100 primary MFG-E8 antibody (Santa Cruz, USA) at 37 ℃ for 1 h, followed by FITC-conjugated secondary antibody at 37 ℃ for 30 min. Next, Dil to stain the membrane and DAPI for the nucleus were applied at 37 ℃ for 10 min. Finally, images were captured and analyzed using a Nikon ECLIPSE Ti camera and NIS Elements software (Nikon).

2.6. Bioinformatics analysis of data mining in the Kaplan-Meier plotter

The Kaplan-Meier plotter is able to assess the effect of 54,675 genes on survival using 10,461 cancer samples. It includes 5143 breast cancer patients in the Kaplan-Meier plotter browser. The data for *MFG-E8* expression were derived from the Kaplan-Meier plotter, from which original data were downloaded (http://kmplot.com/analysis/index.php?p=service&cancer=breast). The correlation between *MFG-E8* expression and overall survival (OS) and disease-free survival (DFS) was examined by data mining in the Kaplan-Meier plotter with the cutoff of median *MFG-E8* expression.

2.7. Statistical analysis

Analyses were performed using SPSS 16.0 (Chicago, IL, USA). The relationship between MFG-E8 expression and clinical pathological characteristics were compared with Pearson chi-squared tests or Fisher's exact probability tests. Survival probabilities were judged by the Kaplan-Meier method and assessed by a log-rank test. Cox proportional hazards regression models were used to assess the association between potential confounding variables and prognosis (OS or DFS). OS was calculated as the time between the first day of diagnosis and disease-related death. DFS was calculated as the time between the first day of diagnosis and the occurrence of local recurrence or distant metastasis. Probability values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathological characteristics of breast cancer patients

The clinicopathological data for the 166 breast cancer patients was summarized in Table S1. The median age of these patients was 51.4 years (range, 20–78 years). The majority of these patients had a tumor with infiltrating ductal carcinoma (77.7%), < 3 cm in size (59.6%), histological Grade I and II (74.7%), or TNM stage I-II (74.1%). Lymph node metastasis occurred in 97 (58.4%) of 166 patients. ER and PR results were combined jointly as the hormone receptor (HR) status. HR + was defined as either ER + or PR+; HR- was defined as both ER- and PR-. Of the 166 patients, 105 (63.3%), 89 (53.6%), and 105 (63.3%), 127 (76.5) were ER-, PR-, HR- and HER2-positive,

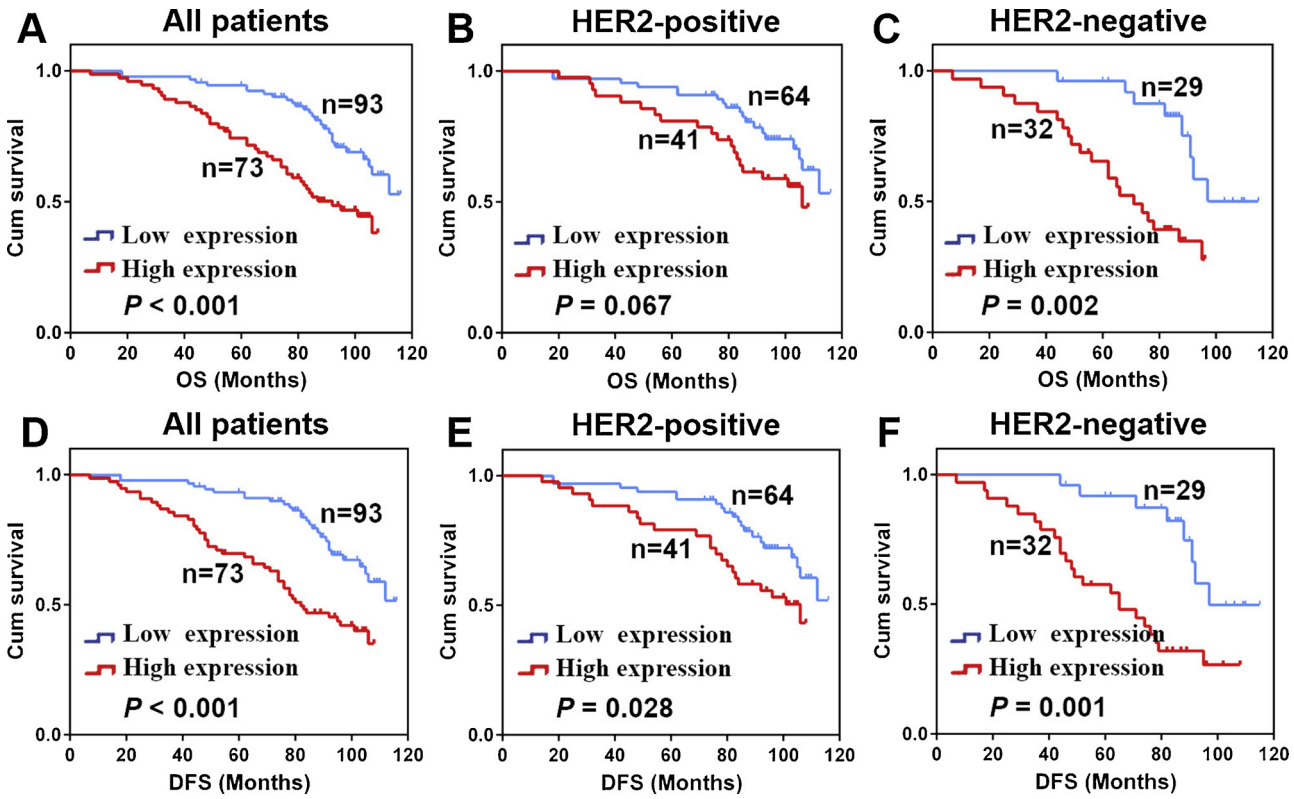

Fig. 3. Kaplan-Meier survival analysis of MFG-E8 expression in breast cancer patients. The log-rank test was performed to evaluate statistical significance. (A) and (D) Survival curves show the association between MFG-E8 expression and OS and DFS in 166 breast cancer patients, respectively (n = 166). (B) and (C) Survival curves show the association between MFG-E8 expression and OS with HER2-positive (n = 105) and HER2-negative status (n = 61). (E) and (F) Survival curves show the association between MFG-E8 expression and DFS with HER2-positive (n = 105) and HER2- negative status (n = 61).

respectively. Follow-up data were available for 166 breast cancer patients. During the follow-up period of 7–116 months, relapses occurred in 73 cases, and cancer-associated deaths were found in 67 cases. The 5-year survival rate was 83.7%. The median OS and DFS was 83.8 months and 81.9 months, respectively.

3.2. MFG-E8 overexpression and location in human breast cancer

We evaluated the expression of MFG-E8 in 166 samples from breast cancer patients and 55 samples from cancer-adjacent control patients, using immunohistochemistry. MFG-E8 immunoreactivity was observed in 73 (44.0%) of 166 breast cancer samples and 6 (10.9%) of 55 control samples. Positive staining of MFG-E8 was observed mainly in the cytoplasm and membrane of the tumor cells. The different immunohistochemical staining images obtained for MFG-E8 in breast cancer and cancer-adjacent tissues are shown in Fig. 1A. MFG-E8 immunoreactivity occurred significantly more frequently in breast cancer than control samples ($P < 0.001$, Fig. 1B, C). Moreover, the Western blot results for 6 pairs of fresh tumor and cancer-adjacent tissues from breast cancer patients demonstrated significantly upregulated MFG-E8 expression in breast cancer samples compared with control samples from the same patient ($P = 0.001$, Fig. 1D, E).

Then, we further explored the localization of MFG-E8 in human breast cancer tissues. Immunofluorescence staining of 6 pairs of fresh tumors and cancer-adjacent tissues showed that MFG-E8 was mainly localized in the cytoplasm and membrane of tumor cells, in agreement with the immunohistochemical staining results (Fig.1F). The fluorescence intensity of MFG-E8 in the breast cancer group (4398 ± 286.1, n = 6) was significantly higher than in the cancer-adjacent group (1046 ± 126.6, n = 6) ($P < 0.001$, Fig. 1G). Based on the above results, we confirmed that MFG-E8 expression was increased in human breast cancer and mainly localized in the cytoplasm and membrane of tumor cells.

3.3. Selection of the cutpoint value for MFG-E8 expression

ROC curve analysis was carried out to determine an optimal cutpoint score for MFG-E8 expression in breast cancer samples. The ROC curves of every clinicopathological characteristic are shown in Fig. 2, which displayed a discrimination of the expression levels of MFG-E8 by TNM stage ($P = 0.004$), histological grade ($P = 0.006$), lymph node metastasis ($P = 0.009$), tumor size ($P = 0.184$), DFS ($P = 0.007$), OS ($P = 0.014$), ER ($P = 0.055$) and HER2 status ($P = 0.316$). The AUC for TNM stage had the biggest area and P value was the smallest of all. Based on this outcome, a cutpoint score of 137.5% was selected for MFG-E8 expression. Tumors with immunohistological scores > 137.5% and ≤ 137.5% were defined as tumors with ‘high” and “low” MFG-E8 expression, respectively; 73 (44.0%) tumors showed high expression of MFG-E8, and 93 (56.0%) tumors exhibited low expression of MFG-E8.

3.4. Association of MFG-E8 expression with the clinicopathological characteristics of breast cancer patients

We then explored the association between MFG-E8 expression and the clinicopathological characteristics of breast cancer. The age, menopausal status, tumor size, histological type, ER status, PR status and HR status of the patients were not significantly associated with the expression of MFG-E8 ($P > 0.05$, Table 1). The high expression levels of MFG-E8 were more associated with lymph node metastasis, TNM stage and histological grade ($P < 0.001$, Table 1).

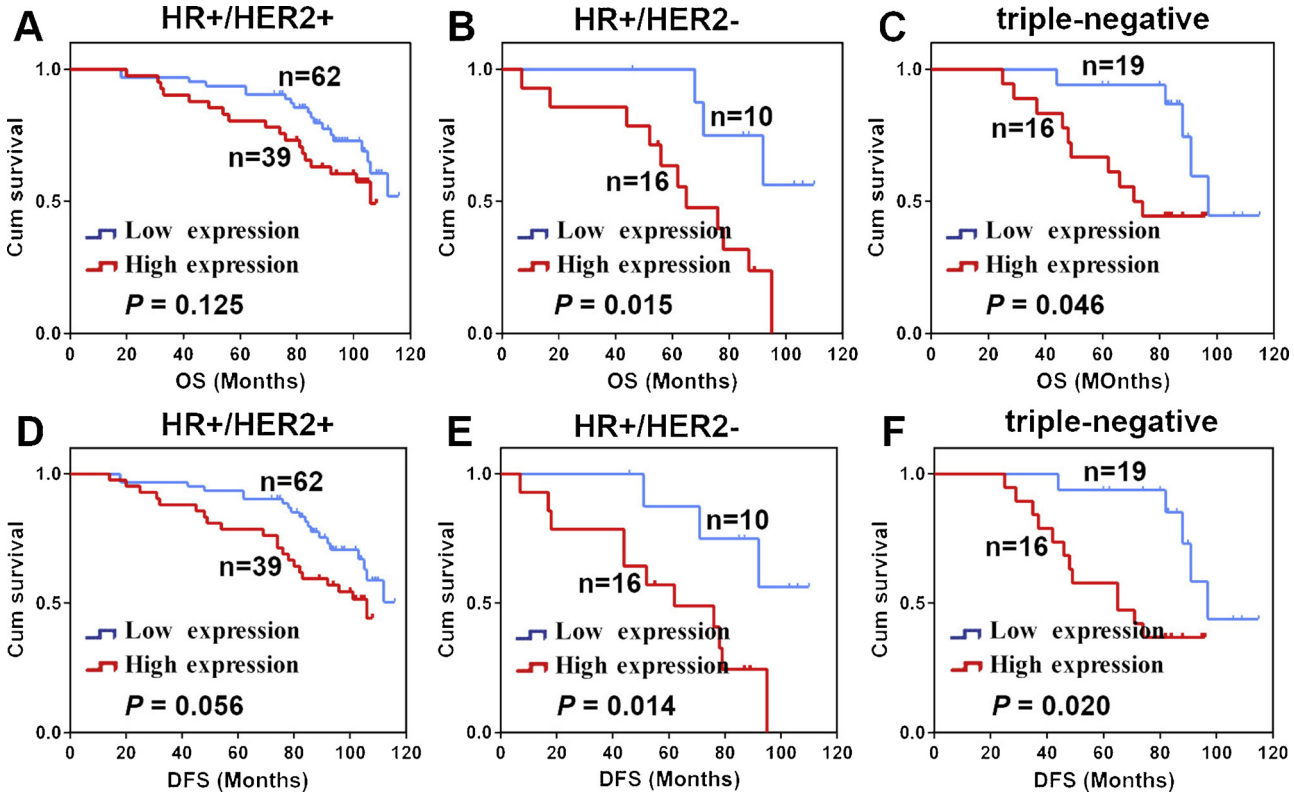

Fig. 4. Survival curves of the association between MFG-E8 expression and OS and DFS in breast cancer patients with different HR and HER2 statuses. ER and PR results were combined jointly as the hormone receptor (HR) status. HR + was defined as either ER + or PR+; HR- was defined as both ER- and PR-. (A)–(C) Survival curves show the association between MFG-E8 expression and OS in HR+/HER2+ patients (A) (n = 101), HR+/HER2- patients (B) (n = 26) and triple-negative patients (C) (n = 35). (D)–(F) Survival curves show the association between MFG-E8 expression and DFS in HR+/HER2+ patients (D) (n = 101), HR+/HER2- patients (E) (n = 26) and triple-negative patients (F) (n = 35). There were only 4 cases patients with HR-/HER2 + .

Table 2
Univariate Cox regression analysis of the association between clinicopathological data and overall survival (OS) and disease-free survival (DFS) in breast cancer patients.

Category	OS		DFS	
	RR[a] (95% CI [b])	*P* [c]	RR (95% CI)	*P*
Age (year) (> 51/≤ 51)	1.175 (0.73˜1.90)	0.511	1.196 (0.75˜1.90)	0.450
Menopausal status (post/pre)	1.087 (0.67˜1.77)	0.735	1.173 (0.73˜1.88)	0.508
Tumor size(cm) (> 3/≤ 3)	1.909 (1.18˜3.09)	**0.008**	1.887 (1.19˜3.00)	**0.007**
Histological grade (III/II/I)	2.725 (1.62˜4.58)	**< 0.001**	2.948 (1.78˜4.88)	**< 0.001**
Histological type (ductal / Lobular / mucinous/Other)	0.971 (0.70˜1.35)	0.861	1.035(0.76˜1.41)	0.829
TNM stage (IV˜III/II/I)	3.090 (1.90˜5.02)	**< 0.001**	3.495 (2.18˜5.58)	**< 0.001**
Lymph node status (≥10/4˜9/1˜3/0)	2.247 (1.32˜3.83)	**0.003**	2.416 (1.44˜4.05)	**0.001**
ER status (positive/negative)	0.568 (0.35˜0.93)	**0.023**	0.570 (0.36˜0.91)	**0.019**
PR status (positive/negative)	0.939 (0.58˜1.52)	0.796	0.942 (0.59˜1.50)	0.800
HR status (positive/negative)	0.813 (0.46˜1.43)	0.472	0.772 (0.45˜1.32)	0.343
HER-2 status (positive/negative)	0.518 (0.32˜0.84)	**0.008**	0.522 (0.33˜0.84)	**0.007**
MFG-E8 (positive/negative)	2.296 (1.40˜3.76)	**0.001**	2.287 (1.42˜3.67)	**0.001**

[a] RR, relative risk.
[b] 95% CI, 95% confidence interval.
[c] *P* value were obtained from Cox proportional hazard analysis.

3.5. Association of MFG-E8 expression with the survival of breast cancer patients

We used Kaplan-Meier analysis to evaluate the association of MFG-E8 expression levels with OS and DFS in human breast cancer patients. Overexpression of MFG-E8 was significantly associated with a shorter OS and DFS in breast cancer patients, respectively (n = 166, $P < 0.001$, $P < 0.001$, Fig. 3A, D). We further explored the association of MFG-E8 expression with OS and DFS in the HER2-positive and HER2-negative group. Our results demonstrated that high expression of MFG-E8 was associated with a shorter OS ($P = 0.002$) and DFS ($P = 0.001$) in patients with HER2-negative breast cancer (Fig. 3B, C, E, F). Next, we combined the ER and PR results jointly as the hormone receptor (HR) status. HR + was defined as either ER + or PR+; HR- was defined as both ER- and PR-. We analyzed the relationship between MFG-E8 and survival time in subgroups of breast cancer patients, categorized according to HR and HER2 status. High MFG-E8 expression was associated with a shorter OS in patients with HR+/HER2- ($P = 0.015$, n = 26) and HR-/HER2- ($P = 0.046$, n = 35) breast cancer (Fig. 4A-C). Moreover, high expression of MFG-E8 was associated with a shorter

Table 3
Multivariate Cox Regression analysis of the association between clinicopathological data and overall survival (OS) and disease-free survival (DFS) in breast cancer patients.

Category	OS		DFS	
	RR[a] (95% CI [b])	*P* [c]	RR (95% CI)	*P*
Tumor size(cm) (> 3/≤ 3)	1.107 (0.63˜1.96)	0.725	1.005 (0.58˜1.75)	0.640
Histological grade (III/II/I)	1.605 (0.57˜4.53)	0.372	1.708 (0.61˜4.75)	0.197
TNM stage (IV˜III/II/I)	2.705(1.63˜4.50)	< **0.001**	3.294 (2.01˜5.40)	< **0.001**
Lymph node status (≥10/4˜9/1˜3/0)	0.800 (0.29˜2.20)	0.665	0.803(0.30˜2.18)	0.493
ER status (positive/negative)	7.515 (0.95˜59.34)	0.056	6.828 (0.88˜52.78)	0.070
HER-2 status (positive/negative)	0.505 (0.31˜0.82)	**0.006**	0.473 (0.29˜0.76)	**0.002**
MFG-E8 (positive/negative)	1.745 (1.04˜2.92)	**0.035**	1.671 (1.02˜2.74)	**0.042**

[a] RR, relative risk.
[b] 95% CI, 95% confidence interval.
[c] *P* value were obtained from Cox proportional hazard analysis.

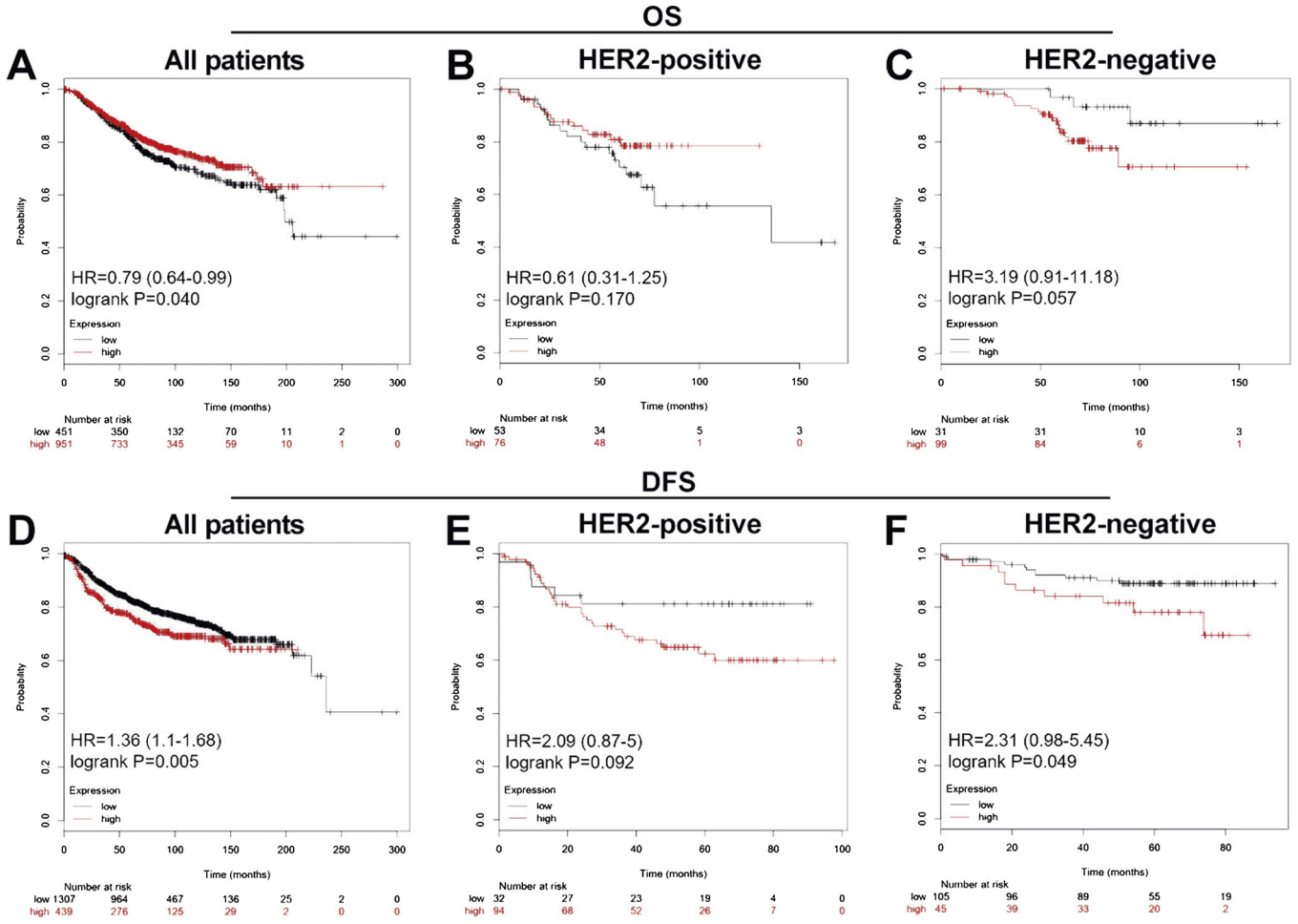

Fig. 5. Bioinformatics analysis of the correlation between *MFG-E8* expression and prognosis in breast cancer using the Kaplan-Meier plotter. All data derived from 5143 cases of breast cancer patients. (A–C) Kaplan-Meier curves of OS with all patients, HER2-positive and HER2- negative groups. (D–F) Kaplan-Meier curves of DFS with all patients, HER2-positive and HER2- negative groups.

DFS in patients with HR + /HER2- ($P = 0.014$, n = 26) and HR-/HER2- ($P = 0.020$, n = 35) breast cancer (Fig. 4D-F). Because there were only 4 cases of patients with HR-/HER2 +, this subgroup was not analyzed.

We then employed a univariate Cox regression analysis to assess the influence of each clinicopathological variable on OS and DFS in human breast cancer patients. The univariate analysis confirmed that tumor size, histological grade, TNM stage, lymph node metastasis, ER status, HER2 status and MFG-E8 were significantly associated with the OS and DFS of breast cancer patients (Table 2). Furthermore, multivariate Cox regression analysis revealed that TNM stage was a predictive prognostic factor for a shorter OS and DFS in breast cancer patients ($P < 0.001$, Table 3). HER2 status was also a predictive prognostic factor for a shorter OS ($P = 0.006$, Table 3) and DFS ($P < 0.002$, Table 3) in breast cancer patients. Notably, we confirmed that MFG-E8 over-expression was a prognostic factor for a shorter OS ($P = 0.035$, Table 3) and DFS ($P = 0.042$, Table 3) in breast cancer patients.

3.6. MFG-E8 expression levels are associated with breast cancer prognosis based on the Kaplan-Meier plotter database

To investigate the influence of *MFG-E8* on breast cancer, we performed the analysis using the Kaplan-Meier plotter browser (http://kmplot.com/analysis/index.php?p= service & cancer = breast). We analyzed the OS and DFS curves of the *MFG-E8* gene family in 5143 cases of breast cancer patients. The log-rank test of the OS curve suggested that *MFG-E8* gene overexpression was remarkably associated with a shorter OS ($P = 0.040$, Fig. 5A) and DFS ($P = 0.005$, Fig.5D) compared with low *MFG-E8* expression. High *MFG-E8* gene expression was also associated with a shorter DFS in the HER2-negative group ($P = 0.049$, Fig. 5F). The gene expression of *MFG-E8* showed no association with either OS ($P = 0.170$, Fig. 5B) or DFS ($P = 0.092$, Fig. 5E) in the HER2-positive group.

4. Discussion

In this study, we showed that the expression of MFG-E8 was significantly upregulated in breast cancer compared with cancer-adjacent tissue. Furthermore, overexpression of MFG-E8 was associated with TNM stage, lymph node metastasis and histological grade. We also found that high MFG-E8 expression resulted in a shorter OS and DFS. Furthermore, TCGA database analysis showed that *MFG-E8* overexpression was markedly associated with a shorter OS compared with low MFG-E8 expression over 5 years.

MFG-E8 is a multifunctional glycoprotein that plays an important role in mediating homeostasis maintenance and immune tolerance and enhancing angiogenesis. Previous studies have reported that MFG-E8 is highly expressed in many tumors, including oral squamous cell carcinoma, colorectal cancer and breast cancer [20,34,35]. Consistent with these studies, we found that MFG-E8 was highly expressed in 73 (44.0%) of 166 breast cancer patients. In contrast, we detected MFG-E8 expression in only 10.9% of the 55 cancer-adjacent samples. We confirmed that MFG-E8 was significantly upregulated in breast cancer compared with cancer-adjacent tissue by immunohistochemistry and Western blot analysis. Immunofluorescence staining confirmed that MFG-E8 was mainly located in the cytoplasm and membrane of tumor cells. These findings suggest that MFG-E8 overexpression may facilitate malignant transformation of normal epithelial cells into breast cancer.

A previous study has reported that MFG-E8 promotes tumor progression in oral squamous cell carcinoma because of the clearance of apoptotic tumor cells by living tumor cells [20]. Min Jia et al also demonstrated a high level of MFG-E8 expression regulation in human colorectal cancer and an association of MFG-E8 overexpression with lymph node metastasis and angiogenesis [33]. Chuanwei Yang's study showed MFG-E8 was upregulated in triple-negative breast cancers as a target gene of the P63 pathway [30]. Although MFG-E8 is overexpressed in human breast cancer, the cause of this overexpression in breast cancers and the relationship between breast cancer and its clinicopathological characteristics remain unclear. In the present study, we showed that high expression levels of MFG-E8 had a great association with TNM stage and lymph node metastasis. These results suggest that overexpression of MFG-E8 predicates more aggressive behavior of human breast cancer.

High expression of MFG-E8 protein has been reported to be an independent poor prognostic factor for melanoma [33]. Overexpression of MFG-E8 is associated with increased metastasis and mortality and poorer differentiation in cholangiocarcinoma [36]. However, the relationship between the expression of MFG-E8 and patient survival in breast cancer has not been addressed until now. In the present study, we found that high expression of MFG-E8 protein was associated with a significantly poorer prognosis in breast cancer patients. The same results were obtained in the HER2-negative, HR+/HER2- and triple-negative groups (Fig. 3,4).

Finally, we performed a bioinformatics analysis of data mining with the Kaplan-Meier plotter. The results showed that *MFG-E8* gene overexpression was markedly associated with a short OS and DFS compared with low MFG-E8 expression (Fig. 5). This result is similar to that reported by C Carracosa, who verified that the *MFG-E8* gene was highly expressed in breast cancer and associated with a shorter survival in ER-negative status patients [35]. Based on the above results, we found that high MFG-E8 expression at both gene and protein levels was associated with a short OS and DFS.

In summary, we detected the expression of MFG-E8 in breast cancer and analyzed the correlation of MFG-E8 expression with the clinicopathological characteristics and prognosis of breast cancer patients. We found that overexpression of MFG-E8 was involved in the TNM stage, lymph node metastasis, histological grade and poor prognosis in breast cancer patients. Moreover, high MFG-E8 expression was associated with a shortened OS and DFS at both the gene and protein level. Our study suggests that MFG-E8 may promote breast cancer tumorigenesis and represents a novel biomarker for prognosis in breast cancer.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China and Liaoning joint fund key program (No. U1608281), National Natural Science Foundation of China (No. 81573462, 81601370), Liaoning Province Scientific Research Foundation (2014226033, LK201618), the Key Laboratory Foundation from Shenyang S&T Projects (F16-094-1-00) and Shenyang S&T Projects (17-123-9-00).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2018.12.036.

References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, et al., Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2) (2015) 87–108.
[2] A.L. Siu, Screening for breast Cancer: u.s. Preventive services task force recommendation statement, Ann. Intern. Med. 164 (4) (2016) 279–296.
[3] H.D. Nelson, M. Pappas, A. Cantor, J. Griffin, M. Daeges, et al., Harms of breast Cancer screening: systematic review to update the 2009 U.S. Preventive services task force recommendation, Ann. Intern. Med. 164 (4) (2016) 256–267.
[4] L. Wein, S.J. Luen, P. Savas, R. Salgado, S. Loi, Checkpoint blockade in the treatment of breast cancer: current status and future directions, Br. J. Cancer (2018).
[5] L. Gerratana, D. Basile, G. Buono, S. De Placido, M. Giuliano, et al., Androgen receptor in triple negative breast cancer: a potential target for the targetless subtype, Cancer Treat. Rev. 68 (2018) 102–110.
[6] M. Rampurwala, K.B. Wisinski, R. O'Regan, Role of the androgen receptor in triple-negative breast cancer, Clin. Adv. Hematol. Oncol. 14 (3) (2016) 186–193.
[7] B. Rahim, R.A.R. O'Regan, Signaling in breast Cancer, Cancers (Basel). 9 (3) (2017).
[8] X. Huang, B. Borgström, J. Stegmayr, Y. Abassi, M. Kruszyk, et al., The molecular basis for inhibition of stemlike Cancer cells by salinomycin, ACS Cent. Sci. 4 (6) (2018) 760–767.
[9] S.P. Bagaria, P.S. Ray, M.S. Sim, X. Ye, J.M. Shamonki, et al., Personalizing breast cancer staging by the inclusion of ER, PR, and HER2, JAMA Surg. 149 (2) (2014) 125–129.
[10] I.M. Miligy, K.L. Gorringe, M.S. Toss, A.A. Al-Kawaz, P. Simpson, et al., Thioredoxin-interacting protein is an independent risk stratifier for breast ductal carcinoma in situ, Mod. Pathol. (2018).
[11] A. Rocca, A. Schirone, R. Maltoni, S. Bravaccini, L. Cecconetto, et al., Progress with palbociclib in breast cancer: latest evidence and clinical considerations, Ther. Adv. Med. Oncol. 9 (2) (2017) 83–105.
[12] Y. Sagara, M. Takada, Y. Ohi, S. Ohtani, S. Kurozumi, et al., Effectiveness of neoadjuvant systemic therapy with trastuzumab for basal HER2 type breast cancer: results from retrospective cohort study of Japan Breast Cancer research Group (JBCRG)-C03, Breast Cancer Res. Treat. (2018).
[13] M. Debiasi, C.A. Polanczyk, P. Ziegelmann, C. Barrios, H. Cao, et al., Efficacy of Anti-HER2 agents in combination with adjuvant or neoadjuvant chemotherapy for early and locally advanced HER2-Positive breast Cancer patients: a network meta-analysis, Front. Oncol. 8 (2018) 156.

[14] M.J. Duffy, N. Harbeck, M. Nap, R. Molina, A. Nicolini, et al., Clinical use of biomarkers in breast cancer: updated guidelines from the European Group on Tumor Markers (EGTM), Eur. J. Cancer 75 (2017) 284–298.
[15] M. Arnedos, M.A. Bayar, B. Cheaib, V. Scott, I. Bouakka, et al., Modulation of Rb phosphorylation and antiproliferative response to palbociclib. The Preoperative-Palbociclib (POP) randomized Clinical Trial, Ann. Oncol. (2018).
[16] J.A. Peterson, C.D. Scallan, R.L. Ceriani, M. Hamosh, Structural and functional aspects of three major glycoproteins of the human milk fat globule membrane, Adv. Exp. Med. Biol. 501 (2001) 179–187.
[17] A. Kamińska, F.J. Enguita, E.Ł. Stępień, Lactadherin: an unappreciated haemostasis regulator and potential therapeutic agent, Vascul. Pharmacol. 101 (2018) 21–28.
[18] H. Ye, B. Li, V. Subramanian, B.H. Choi, Y. Liang, et al., NMR solution structure of C2 domain of MFG-E8 and insights into its molecular recognition with phosphatidylserine, Biochim. Biophys. Acta 1828 (3) (2013) 1083–1093.
[19] P. Laplante, F. Brillant-Marquis, M.J. Brissette, B. Joannette-Pilon, R. Cayrol, et al., MFG-E8 reprogramming of macrophages promotes wound healing by increased bFGF production and fibroblast functions, J. Invest. Dermatol. 137 (9) (2017) 2005–2013.
[20] M. Yamazaki, S. Maruyama, T. Abé, A. Essa, H. Babkair, et al., MFG-E8 expression for progression of oral squamous cell carcinoma and for self-clearance of apoptotic cells, Lab. Invest. 94 (11) (2014) 1260–1272.
[21] A. Das, S. Ghatak, M. Sinha, S. Chaffee, N.S. Ahmed, et al., Correction of MFG-E8 resolves inflammation and promotes cutaneous wound healing in diabetes, J. Immunol. 196 (12) (2016) 5089–5100.
[22] Y. Tan, B. AlKhamees, D. Jia, L. Li, J.F. Couture, et al., MFG-E8 is critical for embryonic stem cell-mediated t cell immunomodulation, Stem Cell Reports 5 (5) (2015) 741–752.
[23] A.M. Rodríguez, M.V. Delpino, M.C. Miraglia, M.M. Costa Franco, P. Barrionuevo, et al., Brucella abortus-activated microglia induce neuronal death through primary phagocytosis, Glia. 65 (7) (2017) 1137–1151.
[24] Y. Hirano, W.L. Yang, M. Aziz, F. Zhang, B. Sherry, et al., MFG-E8-derived peptide attenuates adhesion and migration of immune cells to endothelial cells, J. Leukoc. Biol. 101 (5) (2017) 1201–1209.
[25] L. Hendricks, M. Aziz, W.L. Yang, J. Nicastro, G.F. Coppa, et al., Milk fat globule-epidermal growth factor-factor VIII-derived peptide MSP68 is a cytoskeletal immunomodulator of neutrophils that inhibits Rac1, J. Surg. Res. 208 (2017) 10–19.
[26] B.Z. Li, H.Y. Zhang, H.F. Pan, D.Q. Ye, Identification of MFG-E8 as a novel therapeutic target for diseases, Expert Opin. Ther. Targets 17 (11) (2013) 1275–1285.
[27] X. Wang, L. Hao, H.F. Bu, A.W. Scott, K. Tian, et al., Spherical nucleic acid targeting microRNA-99b enhances intestinal MFG-E8 gene expression and restores enterocyte migration in lipopolysaccharide-induced septic mice, Sci. Rep. 6 (2016) 31687.
[28] K. Yamada, A. Uchiyama, A. Uehara, B. Perera, S. Ogino, et al., MFG-E8 drives melanoma growth by stimulating mesenchymal stromal cell-induced angiogenesis and M2 polarization of tumor-associated macrophages, Cancer Res. 76 (14) (2016) 4283–4292.
[29] F.N. Soki, A.J. Koh, J.D. Jones, Y.W. Kim, J. Dai, et al., Polarization of prostate cancer-associated macrophages is induced by milk fat globule-EGF factor 8 (MFG-E8)-mediated efferocytosis, J. Biol. Chem. 289 (35) (2014) 24560–24572.
[30] C. Yang, T. Hayashida, N. Forster, C. Li, D. Shen, et al., The integrin alpha(v) beta (3-5) ligand MFG-E8 is a p63/p73 target gene in triple-negative breast cancers but exhibits suppressive functions in ER (+) and erbB2(+) breast cancers, Cancer Res. 71 (3) (2011) 937–945.
[31] H. Wu, S. Guan, M. Sun, Z. Yu, L. Zhao, et al., Ano1/TMEM16A overexpression is associated with good prognosis in PR-Positive or HER2-Negative breast Cancer patients following tamoxifen treatment, PLoS One 10 (5) (2015) e0126128.
[32] Z. Yu, M. Sun, F. Jin, Q. Xiao, M. He, et al., Combined expression of ezrin and E-cadherin is associated with lymph node metastasis and poor prognosis in breast cancer, Oncol. Rep. 34 (1) (2015) 165–174.
[33] M. Jia, H. Yao, C. Chen, Y. Wang, H. Wang, et al., Prognostic correlation between MFG-E8 expression level and colorectal Cancer, Arch. Med. Res. 48 (3) (2017) 270–275.
[34] Q. Zhao, L. Xu, X. Sun, K. Zhang, H. Shen, et al., MFG-E8 overexpression promotes colorectal cancer progression via AKT/MMPs signalling, Tumour Biol. 39 (6) (2017) 1010428317707881.
[35] C. Carrascosa, R.G. Obula, E. Missiaglia, H.A. Lehr, M. Delorenzi, et al., MFG-E8/lactadherin regulates cyclins D1/D3 expression and enhances the tumorigenic potential of mammary epithelial cells, Oncogene 31 (12) (2012) 1521–1532.
[36] Z. Wu, T. Boonmars, I. Nagano, W. Loilome, P. Yongvanit, et al., Milk fat globule epidermal growth factor 8 serves a novel biomarker of opisthorchiasis-associated cholangiocarcinoma, Tumour Biol. 35 (3) (2014) 1985–1995.