

Malic enzyme 1 may be a novel target of breast cancer metastasis

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Abstract

Background Malic enzyme 1 (ME1) catalyzes malate to pyruvate and thus promotes glycolysis, playing a part in the Warburg effect. It also has a potential role in tumor progression, but its function in breast cancer remains to be fully clarified. This work aimed to investigate the prognostic value of ME1 and its possible mechanism in breast cancer.

Methods We evaluated ME1 expression in 220 early breast cancer patients with tissue microarray-based immunohistochemistry and explored the relationships between ME1 expression and clinicopathological features. Survival analyses were further performed to determine its prognostic value. The public database was used to confirm tissue microarray results. Moreover, we profiled ME1 expression in breast cancer cell lines via western blotting, and then assessed it in cell viability and motility via Cell counting kit-8 (CCK-8), colony formation, transwell migration and invasion assays. Reactive oxygen species (ROS) was detected by dihydroethidium (DHE) and 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA).

Results In breast cancer tissues, high ME1 expression was significantly associated with larger tumor size, more lymph node metastasis and more extensive lymph-vascular invasion. Survival analysis showed high ME1 expression was significantly correlated with worse recurrence free survival (RFS). Multivariate analysis further identified high ME1 expression as an independent prognostic factor for RFS, which was confirmed by mRNA results in the public database. In vitro, human epidermal growth factor receptor-2 positive and triple negative breast cancer cell lines showed higher expression of ME1, while Luminal cell lines showed lower expression of ME1. Upregulation of ME1 by transfecting MCF-7 cells with virus vector remarkably enhanced viability and motility, epithelial-mesenchymal transition (EMT), and decreased ROS levels, whereas knockdown of this gene in MDA-MB-468 cells produced totally opposite effects as expected. More important, when pretreated with hydrogen peroxide, an oxidizing agent, MCF-7 cells overexpressing ME1 lost its motility, whereas MDA-MB-468 cells with knock-down of ME1 restored its motility when pretreated with N-acetyl cysteine, an antioxidant.

Conclusions To our knowledge, these clinical and experiment work first suggested that ME1 may be a potential therapeutic target for breast cancer metastasis, and its biological effect is mainly controlled by manipulating ROS.

Background

Breast cancer is the most common malignancy in women and one of the three most common malignant tumors worldwide (1). Surgery, radiation, chemotherapy, endocrine therapy and anti-HER2 targeting therapy are applied for early stage patients depending on clinical features and specific molecular subtype (2). Nonetheless, even in patients with no lymph node metastasis, nearly 20% of them suffer from breast cancer recurrence (3). In addition, breast cancer is estimated to rank second only to lung cancer as the leading cause of cancer death in women (15% of all cancer deaths) in America (4). Therefore, it is

necessary to further characterize patients with high recurrence risk and explore the molecular mechanism of breast cancer progression to help develop personalized treatment.

Reprogramming energy metabolism is one of the hallmarks of cancer, which is known as Warburg effect: cancer cells prefer glycolysis even in the presence of oxygen (5). It has been well accepted that cancer cells reprogram the metabolic patterns to satisfy their rapid proliferation and metastasis (6). Malic enzyme 1 (ME1) is a cytosolic metabolic enzyme that catalyzes the citric acid cycle intermediate malate to pyruvate, the substrate of glycolysis, and converts Nicotinamide adenine dinucleotide phosphate (NADP) to Nicotinamide adenine dinucleotide phosphate hydrate (NADPH), the necessities of various biosynthetic reactions. Besides, ME1 can form physiological complexes with 6-Phosphogluconate dehydrogenase and further increase the pentose phosphate pathway flux and NADPH generation (7). Hence, ME1 promotes glycolysis and is involved in reactive oxygen species (ROS) homeostasis, glutamine metabolism and lipogenesis (8), playing an important role in Warburg effect.

Recently, the potential function of ME1 has been revealed in tumor progression. It has been reported that knockdown of ME1 leads to increased ROS level, upregulated cell apoptosis, slower cell growth and reductive metastasis in gastric cancer (9). ME1 induces epithelial-mesenchymal transition and is associated with a worse prognosis in hepatocellular carcinoma (10) and oral squamous cell carcinoma (11). In addition, downregulation of ME1 activates the tumor suppressor gene p53 which further inhibits ME1 in a positive-feedback, modulating metabolism and senescence (8). Since there is a lacking of research on the role of ME1 in breast cancer, we performed data mining from the public Oncomine database and found that ME1 was upregulated in various cancer types including breast cancer. These findings prompted us to assess the value of ME1 in breast cancer.

In the present study, we used tissue microarray to determine ME1 expression and then analyzed the relationships between ME1 expression and clinicopathological parameters as well as its prognostic value in breast cancer patients. Next, we profiled the expression of ME1 in diverse breast cancer cells and investigated its role in viability and motility besides its impact on ROS level. Our clinical and experimental work identified ME1 as a novel prognostic indicator, highlighted its potential role in the development of breast cancer and found its effect could be mainly blocked by manipulating ROS.

Methods

Patients, tissue specimens and clinical data. The study cohort consisted of 220 female breast cancer patients who underwent radical surgery between 11 August 2015 and 17 May 2016 in Fudan University Shanghai Cancer Center (FUSCC, Shanghai, China). Eligible patients were women who had histologically confirmed invasive breast cancer; had no evidence of distant metastasis; and provided sufficient tissues for further research. Patients were ineligible if they had received neoadjuvant chemotherapy or radiation therapy. Clinical data of the patients was retrieved from the Outcome unit. We completed the follow-up on 26 June 2018 and the medium follow-up period was 29.2 months (range 0.50–34.25). Recurrence free

survival (RFS) time was calculated from the date of radical surgery to the date of breast cancer recurrence (ipsilateral breast, local-regional, or distant), death or the last time of follow-up.

Immunohistochemistry (IHC). Paraffin-embedded tissues were made into tissue microarray. After deparaffinized and rehydrated, the sections were heated in an autoclave at 120°C in sodium citrate buffer (pH 6.0) for 10 min for antigen retrieval. The sections were then incubated with 3% hydrogen peroxide for 15 min. After blocking of nonspecific binding with QuickBlock™ Blocking Buffer (Beyotime, P0260, China) for 15 min, the sections were incubated at 4°C with ME1 antibody (Abcam, ab97445, 1:1000, USA) overnight. The IHC Kit (GTVision, GK500705, China) including second antibody and DAB substrate was used. After washing with PBS, the sections were incubated with the second antibody for 30 min at room temperature. Color was developed with DAB (1:100) for 2 min. The sections were counterstained with hematoxylin (Servicebio, G1004, China), and finally dehydrated.

Immunohistochemical staining score. The standards for IHC staining scoring was previously described (12). Herein the intensity range was 0 = negative; 1 = low; 2 = medium and 3 = high. The quantity 0 = no positivity; 1 = positivity in 0–10%; 2 = positivity in 11–50%; 3 = positivity in 51–80%; 4 = positivity in > 80%. The final immunoreactive score (IRS, ranging from 0–12) was obtained by multiplying the intensity score and the quantity score. Two pathologists blinded to the patients' information scored the immunohistochemical staining. In discrepant cases, they further reviewed the cases and reached a consensus. For ME1 low and high expression was defined as $IRS \leq 6$ and $IRS > 6$, respectively.

Cell lines and cell culture. Cell lines including MCF-7, MDA-MB-468, SKBR3, MDA-MB-231, ZR-75-1 and 293FT were obtained from the Cell Research Institute (Shanghai, China). Cells were routinely cultured in high-glucose DMEM (MCF-7, SKBR3, ZR-75-1 and 293FT) (Hyclone, USA) or L-15 (MDA-MB-468 and MDA-MB-231) (Hyclone, USA), supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) at 37°C in a humidified 5% CO₂ atmosphere (MCF-7, SKBR3, ZR-75-1 and 293FT) or 100% atmosphere (MDA-MB-468 and MDA-MB-231).

Protein extraction and Western blotting analysis. Cells were washed twice with pre-chilled PBS, pelleted by centrifugation and lysed in RIPA (Beyotime, PC102, China). After incubation for 30 min on ice, lysates were centrifuged (12,000g, 10 min, 4°C). Supernatants were collected and the protein concentration was measured using a BCA protein assay reagent (Enzyme, ZJ101, China). Total protein (20ug) was separated on 10% SDS-PAGE and then transferred to nitrocellulose membranes for 2h at room temperature. The membranes were incubated with the appropriate primary antibodies (anti-ME1, Abcam, ab97445, 1:1000, USA; anti-β-actin, Proteintech, 60008-1-Ig, 1:1000, USA) overnight at 4°C, washed three times with TBST, and then incubated with the corresponding secondary antibody (goat anti rabbit IgG, SA00001-2, 1:1000; goat anti mouse IgG, SA00001-1, 1:1000, Proteintech, USA) for 1h at room temperature. The bands were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0100, USA) and detected with a luminescent image analyzer (GE Image Quant LAS4000 mini, USA).

Plasmids and lentivirus infection. The shRNA plasmids for negative control and ME1 were purchased from Genechem Company (Shanghai, China). The ectopic overexpression plasmids for negative control and ME1 were purchased from XY biotech Company (Shanghai, China). Lentivirus carrying ME1 cDNA or shRNA targeting ME1 was produced from 293FT cells. MCF-7 and MDA-MB-468 cells were infected with lentivirus and then selected with puromycin. The overexpression and knockdown efficacy were evaluated by Western blotting.

Cell viability assays. Cells were seeded in 96-well plates (2000 cells/well) in triplicate and cell viability was examined by Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Japan). Cells were seeded in 6-well plates (1000 cells/well) in triplicate and cultured for two weeks in colony-formation assay. Colonies were washed three times in PBS, fixed with 4% formaldehyde and stained with Crystal violet for 5 min.

Cell motility assays. The migration and invasion assays were done in a 24-well Chemotaxis chamber with 8- μ m pores (Corning, USA). For migration assays, 5×10^4 cells were seeded into the Matrigel-uncoated upper insert at 24-well in medium without serum. Medium containing 20% serum was added to the well as a chemoattractant. Following a culture of 36 h, non-invading cells were removed from the upper surface by wiping with a cotton swab. For invasion assays, 1×10^6 cells were seeded into the Matrigel-coated upper insert at 24 wells in medium without serum. Medium containing 20% serum was added to the well as a chemoattractant. Following a culture of 48 h, non-invading cells were removed from the upper surface by wiping with a cotton swab. Then, the membranes were fixed with 4% formaldehyde for 15 min. The invading cells were stained with Crystal violet for 5 min.

Detection of ROS level. Cells in 6-well plates were washed with PBS three times and incubated with Dihydroethidium (DHE, Beyotime, S0063, China). or 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime, S0033, China) for 30 min at room temperature. Afterwards, cells were collected, washed with PBS three times and resuspended in PBS. DHE or DCF fluorescence was measured by FACScan Flow Cytometer (Beckman-Coulter, USA) within 30 min.

Statistical analyses. We evaluated the correlations between ME1 expression and clinicopathological parameters by Pearson Chi-square test or Fisher's exact test. Recurrence free survival was plotted and calculated using Kaplan-Meier (KM) curve while differences between groups were compared by log-rank test. Univariate Cox proportional hazard model was used to estimate the influence of each variable on RFS. Variables with P values < 0.1 were further included in multivariate Cox proportional hazard models. In laboratory experiments, quantitative variables were illustrated as means \pm SD and analyzed with the Student's t test. Two-sided P values ≤ 0.05 were considered statistically significant. All analyses were performed by SPSS 22.0.

Results

ME1 expression in breast cancer tissues

To confirm the role of ME1 in breast cancer, we first performed IHC staining of ME1 in a tissue microarray containing 220 early stage breast cancer patients. We confirmed the specificity of the antibody via ME1 knockdown (see Figure 4F). Interstitial tissue was taken as negative control. Several representative cases are shown in Figure 1A–1D. We evaluated ME1 protein expression semi-quantitatively. As defined above, 51.8% (114/220) and 48.2% (106/220) of the patients were categorized as ME1-high and ME1-low cases, respectively.

Associations between ME1 expression and clinicopathological parameters

Next, we explored the correlations between ME1 expression by IHC and clinicopathological parameters in our cohort (Table 1). Comparing to ME1-low cases, ME1-high cases were significantly associated with larger tumor size ($P = 0.036$), more lymph node metastasis ($P < 0.001$) and more extensive lymph-vascular invasion ($P = 0.001$). However, we did not find any specific relations of ME1 expression with age, histopathologic type, histologic grade, ki67 index or molecular subtype, partially due to the limited sample size of our cohort. Moreover, we investigated the correlation between ME1 mRNA level and clinicopathological parameters in Curtis Breast dataset via Oncomine database (13). High ME1 mRNA level correlated to higher histologic grade, later TNM stage, TNBC and HER2 positive breast cancer (see Figure 2A). In short, high expression of ME1 in both the mRNA and protein levels was associated with risk factors of breast cancer recurrence.

Associations between ME1 expression and clinical outcomes

Since ME1 expression correlated with risk factors of breast cancer recurrence, we further explored its relationship with clinical outcomes. Herein, we defined RFS as date from radical surgery to first recurrence of breast cancer (ipsilateral breast, local-regional, or distant), death or the last time of follow-up and evaluated the association between ME1 expression and RFS in our cohort. Survival analysis by the log-rank test showed high ME1 expression was significantly correlated with worse RFS ($P < 0.01$) (see Figure 2B). In addition, univariable cox regression showed pathologic N stage ≥ 2 , lymph-vascular invasion and ME1 high expression were associated with worse RFS ($P < 0.1$). Further multivariable cox regression identified ME1 high expression as an independent negative prognostic factor for RFS (HR = 5.343, 95% CI = 1.191–23.971, $P = 0.029$) (Table 2). Moreover, we conducted stratified analysis which revealed high ME1 expression was related to worse RFS among cases more than 45 years old (HR = 10.725, 95% CI = 1.394–82.482, $P = 0.023$) and among those with Ki67 index $\geq 20\%$ (HR = 4.127, 95% CI = 1.176–14.485, $P = 0.027$) (see Figure 3).

Finally, we investigated the relationships between ME1 mRNA expression and survival in breast cancer via an online tool KM plotter (14). Consistently, high ME1 mRNA expression related to both worse RFS

(HR = 1.29, 95% CI = 1.04–1.59, $P < 0.05$) (see Figure 2C) and worse overall survival (HR = 1.39, 95% CI = 1.24–1.55, $P < 0.05$) (see Figure 2D). Collectively, high expression of ME1 indicated worse prognosis of breast cancer patients.

ME1 expression in breast cancer cells

Due to the correlations between ME1 expression with clinicopathological features and outcomes, we speculated that ME1 could participate in breast cancer progression and further explore its functions *in vitro*. To understand the differential expression of ME1 in breast cancer cell lines with diverse malignancy, ME1 protein level was detected in several representative cell lines by western blotting assays. ME1 showed higher expression in the more aggressive cell lines including TNBC cell lines (MDA-MB–468 and MDA-MB–231) and HER2 positive cell line (SKBR3), while lower expression in luminal cell lines (ZR–75–1 and MCF–7) (see Figure 4A).

Effect of ME1 on cell viability, motility and EMT in breast cancer cells

To determine the effects of ME1 in breast cancer cells, we constructed MCF–7 cell line stably overexpressing ME1 and MDA-MB–468 cell line stably expressing shRNA against ME1. The overexpressing and silencing effect were verified via western blotting assays (see Additional file 1). We first conducted cell viability assays. Ectopic expression of ME1 in MCF–7 cells significantly accelerated cell proliferation in CCK–8 assays and increased colony formation in colony growth assays (see Additional file 2). On the contrary, cell proliferation and clonogenicity were suppressed with knockdown of ME1 in MDA-MB–468 cells (see Additional file 2). Next, we examined whether ME1 could influence the ability of cell migration and invasion, which is another distinct feature of malignant tumor. Overexpression of ME1 in MCF–7 cells enhanced cell motility in both transwell migration and invasion assays (see Figure 4B and 4C), accompanied by the development of epithelial-mesenchymal transition (EMT) (see Figure 4F). In contrast, knockdown of ME1 in MDA-MB–468 cells had a profound inhibitory effect on cell migration, invasion and EMT (see Figure 4D, 4E and 4F).

ME1 promotes breast cancer motility via ROS regulation

It is acknowledged that ME1 catalyzes malate to pyruvate, accompanied by NADP⁺ converting to NADPH, which plays crucial role on ROS homeostasis. Thus, we assessed ROS level to explore the possible mechanism by which ME1 could promote breast cancer progression. In accordance with our speculation, DHE fluorescence revealed that ME1 overexpression significantly decreased ROS level in MCF–7 cells (see Additional file 3). Oppositely, ME1 knockdown apparently increased ROS level in MDA-MB–468 cells by DCF fluorescence (see Additional file 3). When pretreated with hydrogen peroxide, an oxidizing agent, MCF–7 cells overexpressing ME1 showed higher ROS level and lost its motility in transwell migration

assays (see Figure 4A, 4C and 4D). Moreover, MDA-MB-468 cells with knock-down of ME1 showed lower ROS level and restored its motility when pretreated with N-acetyl cysteine, an antioxidant (see Figure 4B, 4E and 4F).

Discussion

In the present study, we demonstrated that ME1 high expression was associated with risk factors of breast cancer recurrence and indicated worse prognosis. Thus, we found out the important role of ME1 in breast cancer. In addition to regular risk factors, clinicians can further distinguish high recurrence-risk patients after radical surgery according to ME1 expression and possibly develop suitable treatment for these patients. Further work showed ME1 promoted cell viability, motility and EMT in breast cancer cells, probably by decreasing intracellular ROS level, which probably reflects ROS level in the tumor micro-environment.

Previous study of ME1 in breast cancer only focusing on TNBC subtype drew the same conclusion as ours that ME1 was high expressed in TNBC cells (15), but its upstream up-regulators have not been reported yet. We also found the relationship between ME1 expression and HER2 positive in Curtis Breast Dataset. This phenomenon was supported by the evidence that NR1D1, the positive transcription factor of ME1, resides on ERBB2-containing 17q12-21 amplicons and is part of the ERBB2 expression signature (16).

The role of ME1 in breast cancer has not been fully elucidated. Although ME1 is reported to enhance cell proliferation and metastasis capacity in multiple cancer types (10, 11, 15, 17), the underlying mechanism of which still remains inexplicit. On one hand, ME1 catalyzes malate to pyruvate, inducing cell glucose uptake and lactate production and thus promote Warburg effect, which is favorable for tumor (15). On the other hand, NADPH generated from ME1-catalyzed reaction reduces ROS accumulation, which is the common byproduct during tumor progression. Otherwise, excessive ROS can result in macromolecules damage including lipids, proteins, and nucleic acids, which is unfavorable for tumor (18). Moreover, ROS released to micro-environment may lead to ROS activation in other cell types including fibroblasts, vascular endothelial cells, immune cells and etc., which can in turn have a crosstalk with tumor cells. Our study implied that ME1 could help maintain the homeostasis of ROS and partially explained the function of ME1, in accordance with the results in gastric cancer cells (9).

As far as we know, there is still no anti-tumor drugs targeting ME1. Considering the important role of ME1 in tumor progression, we also reflect on whether ME1 is a potential therapeutic target. Since ME1 is widely expressed in human tissues and it proves essential for the survival of normal fibroblasts (19), ME1 inhibition might do harm to normal tissues severely and may not be a suitable therapeutic strategy. As demonstrated above, ME1 is highly expressed in HER2 positive cancer, hence ME1 inhibition conjugated to trastuzumab could be a possible treatment for HER2 positive patients. Alternatively, targeting glycolysis, NADPH-producing enzymes, redox-regulating enzymes (20) or potential downstream molecules of ME1 may be a better approach.

There are some limitations in our study. The follow-up period of our cohort was still short and the recurrence rate was only 7.7% (17/220) by the end-up point of our study, but our results were supported by data from KM plotter with a longer follow-up period. In order to identify whether ME1 influence the efficacy of treatment, information of adjuvant therapy including chemotherapy, anti-HER2 therapy and radiotherapy requires to be updated, which is ongoing in our center. Besides, we did not find the exact molecular mechanisms by which ME1 promoted tumor progression, which is now in active research in our laboratory. Despite these limitations, this study can clearly indicate the important role of ME1 in breast cancer and give emphasis on the relationship between Warburg effect and breast cancer.

Conclusion

This study suggested that ME1 correlated with worse prognosis and enhanced cell progression via manipulating micro-environment ROS in breast cancer. More research is guaranteed to prove that ME1 is a potential therapeutic target for breast cancer.

Abbreviation

HER2 Human epidermal growth factor receptor-2

ER Estrogen receptor

ME1 Malic enzyme 1

NADP Nicotinamide adenine dinucleotide phosphate

NADPH Nicotinamide adenine dinucleotide phosphate hydrate

ROS Reactive oxygen species

IHC Immunohistochemistry

IRS Immunoreactive score

shRNA Short hairpin RNA

CCK-8 Cell counting kit-8

DHED Hydroethidium

DCFH-DA 2',7'-Dichlorodihydrofluorescein diacetate

KM Kaplan-Meier

RFS Recurrence free survival

TNBC Triple negative breast cancer

HR Hazard ratio

CI Confidence interval

Declarations

Ethics approval and consent to participate This study was approved by ethics committee of Fudan University Shanghai Cancer Center. Written informed consent were obtained from all patients to utilize tissues for research. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication Not applicable.

Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests The authors declare that they have no competing interests

Funding Not applicable.

Authors' contributions Hu XC conceived, designed the study and revised the manuscript. Liu C performed the experiments in laboratory and wrote the manuscript. Lin SC and Zhao YN did data analyses. Cao J and Tao ZH managed the clinical data and follow-ups of the patients. All authors read and approved the final manuscript for publication.

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Tables

Table 1. Clinicopathological parameters of patients and ME1 protein expression

| Parameter | No. of patients n=220 | ME1 expression | | P-value |
|--------------------------------|-----------------------|----------------|------------|--------------|
| | | Low n=106 | High n=114 | |
| Age, n (%) | | | | 0.274 |
| < 45 years | 53(24.1) | 29(27.4) | 24(21.1) | |
| ≥ 45 years | 167(75.9) | 77(72.6) | 90(78.9) | |
| Tumor size, n (%) | | | | 0.036 |
| ≤ 2cm | 86(39.1) | 49(46.2) | 37(32.5) | |
| > 2cm | 134(60.9) | 57(53.8) | 77(67.5) | |
| Lymph node metastasis, n (%) | | | | 0.001 |
| No | 113(51.4) | 68(64.2) | 45(39.5) | |
| Yes | 107(48.6) | 38(35.8) | 69(60.5) | |
| Histopathologic type, n (%) | | | | 0.941 |
| IDC | 201(91.4) | 97(91.5) | 104(91.2) | |
| Non-IDC | 19(8.6) | 9(8.5) | 10(8.8) | |
| Histologic grade, n (%) | | | | 0.907 |
| 1-2 | 74 (33.6) | 37 (34.9) | 37 (32.5) | |
| 3 | 132 (60.0) | 62 (58.5) | 70 (61.4) | |
| Unknown | 14 (6.4) | 7 (6.6) | 7 (6.1) | |
| Ki67%, n (%) | | | | 0.787 |
| < 20% | 22(10.0) | 10(9.4) | 12(10.5) | |
| ≥ 20% | 198(90.0) | 96(90.6) | 102(89.5) | |
| Lymph-vascular invasion, n (%) | | | | 0.001 |
| Negative | 113 (51.4) | 66 (62.3) | 47 (41.2) | |
| Positive | 106 (48.2) | 39 (36.8) | 67 (58.8) | |
| Unknown | 1 (0.5) | 1 (0.9) | 0 (0.0) | |
| HR status, n (%) | | | | 0.435 |
| Positive | 104(52.7) | 53(50.0) | 51(44.7) | |
| Negative | 116(52.7) | 53(50.0) | 63(55.3) | |
| HER2 status, n (%) | | | | 0.184 |
| Positive | 55 (25.0) | 21 (19.8) | 34 (29.8) | |
| Negative | 162 (73.6) | 83 (78.3) | 79 (69.3) | |
| Unknown | 3 (1.4) | 2 (1.9) | 1 (0.9) | |
| Molecular subtype, n (%) | | | | 0.190 |
| Luminal A | 15(6.8) | 6(5.7) | 9(7.9) | |
| Luminal B | 89(40.5) | 47(44.3) | 42(36.8) | |
| HER2 positive | 24(10.9) | 7(6.6) | 17(14.9) | |
| TNBC | 92(41.8) | 46(43.4) | 46(40.4) | |
| Adjuvant chemotherapy, n (%) | | | | 0.953 |
| Yes | 190 (86.4) | 91 (85.8) | 99 (86.8) | |
| No | 21 (9.5) | 10 (9.4) | 11 (9.6) | |
| Unknown | 9 (4.1) | 5 (4.7) | 4 (3.5) | |

$P \leq 0.05$ is considered statistically significant. Significant P -values are in bold.

HR status was evaluated using immunohistochemistry. HER2 status was evaluated using both immunohistochemistry and fluorescence *in situ* hybridization.

Abbreviations: ME1, Malic enzyme 1; IDC, Invasive ductal carcinoma; HR, Hormone receptor; HER2, Human epidermal receptor-2; TNBC, Triple negative breast cancer.

Table 2. Survival analysis of RFS by Cox regression

| Parameter | Univariate | | | Multivariate | | |
|--------------------------|------------|----------------|-----------------|--------------|----------------|-----------------|
| | HR | 95% CI | <i>P</i> -value | HR | 95% CI | <i>P</i> -value |
| Age | | | | | | |
| ≥ 45 years | 1 | | | | | |
| < 45 years | 0.962 | [0.314–2.952] | 0.946 | | | |
| Tumor size | | | | | | |
| ≤ 2cm | 1 | | | | | |
| > 2cm | 1.217 | [0.450–3.291] | 0.699 | | | |
| Pathologic N stage | | | | | | |
| pN0 | 1 | | | | | |
| pN1-3 | 1.963 | [0.726–5.310] | 0.184 | | | |
| Pathologic N stage | | | | | | |
| pN0-1 | 1 | | | 1 | | |
| pN2-3 | 3.505 | [1.352–9.086] | 0.010 | 1.731 | [0.549–5.463] | 0.349 |
| Histopathologic type | | | | | | |
| Non-IDC | 1 | | | | | |
| IDC | 0.383 | [0.110–1.333] | 0.132 | | | |
| Histologic grade | | | | | | |
| 1-2 | 1 | | | | | |
| 3 | 1.353 | [0.470–3.897] | 0.575 | | | |
| Ki67% | | | | | | |
| < 20% | 1 | | | | | |
| ≥ 20% | 1.862 | [0.247–14.048] | 0.547 | | | |
| HR status | | | | | | |
| Negative | 1 | | | | | |
| Positive | 0.545 | [0.201–1.477] | 0.233 | | | |
| HER2 status | | | | | | |
| Negative | 1 | | | | | |
| Positive | 0.931 | [0.303–2.855] | 0.900 | | | |
| Molecular classification | | | | | | |
| Luminal A | 1 | | 0.602 | | | |
| Luminal B | 0.789 | [0.092–6.759] | 0.829 | | | |
| HER2+ | 2.026 | [0.211–19.490] | 0.541 | | | |
| TNBC | 1.367 | [0.171–10.950] | 0.768 | | | |
| Lymph-vascular invasion | | | | | | |
| Negative | 1 | | | 1 | | |
| Positive | 3.392 | [1.094–10.519] | 0.034 | 1.895 | [0.504–7.122] | 0.344 |
| Adjuvant chemotherapy | | | | | | |
| No | 1 | | | | | |
| Yes | 0.811 | [0.185–3.550] | 0.781 | | | |
| ME1 expression | | | | | | |
| Low | 1 | | | 1 | | |
| High | 4.482 | [1.288–15.597] | 0.018 | 5.343 | [1.191–23.971] | 0.029 |

$P \leq 0.05$ is considered statistically significant. Significant P -values are in bold.

HR status was evaluated using immunohistochemistry. HER2 status was evaluated using both immunohistochemistry and fluorescence *in situ* hybridization.

Abbreviations: RFS, Recurrence free survival; HR, Hazard ratio; CI, Confidence interval; IDC, Invasive ductal carcinoma; HR, Hormone receptor; HER2, Human epidermal receptor-2; TNBC, Triple negative breast cancer; ME1, Malic enzyme 1.

Figures

Figure 1

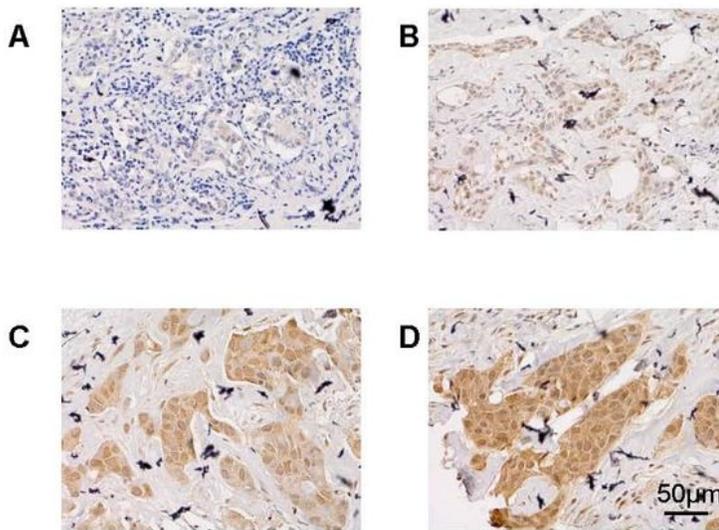


Figure 1

Representative immunohistochemistry images in breast cancer tissues with ME1 staining (magnification, 400×). (A) IRS = 1, ME1-low; (B) IRS = 6, ME1-low; (C) IRS = 8, ME1-high; (D) IRS = 12, ME1-high. Abbreviations: ME1, Malic enzyme 1; IRS, Immunoreactive score.

Figure 2

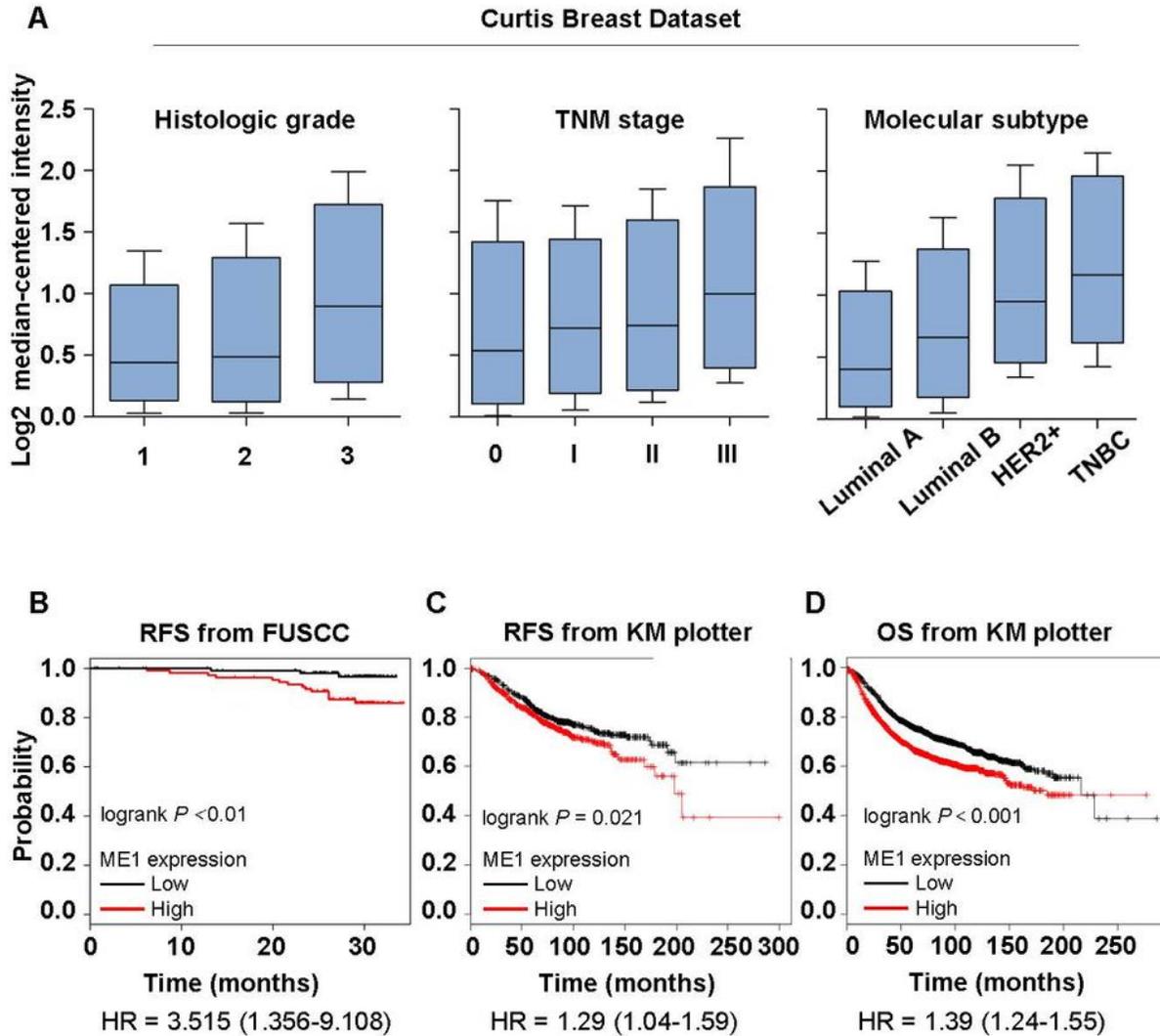


Figure 2

ME1 high expression correlated with risk factors of breast cancer recurrence and indicated worse prognosis. (A) ME1 mRNA expression with histologic grading, TNM staging and molecular subtype (data from Curtis Breast dataset, Oncomine database); (B) KM test for RFS stratified by ME1 protein level (data from FUSCC); (C) KM test for RFS stratified by ME1 mRNA level (data from KM plotter); (D) KM test for overall survival stratified by ME1 mRNA level (KM plotter). Abbreviations: ME1, Malic enzyme 1; KM, Kaplan-Meier; RFS, Recurrence free survival; HR, Hazard ratio; FUSCC, Fudan University Shanghai Cancer Center.

Figure 3

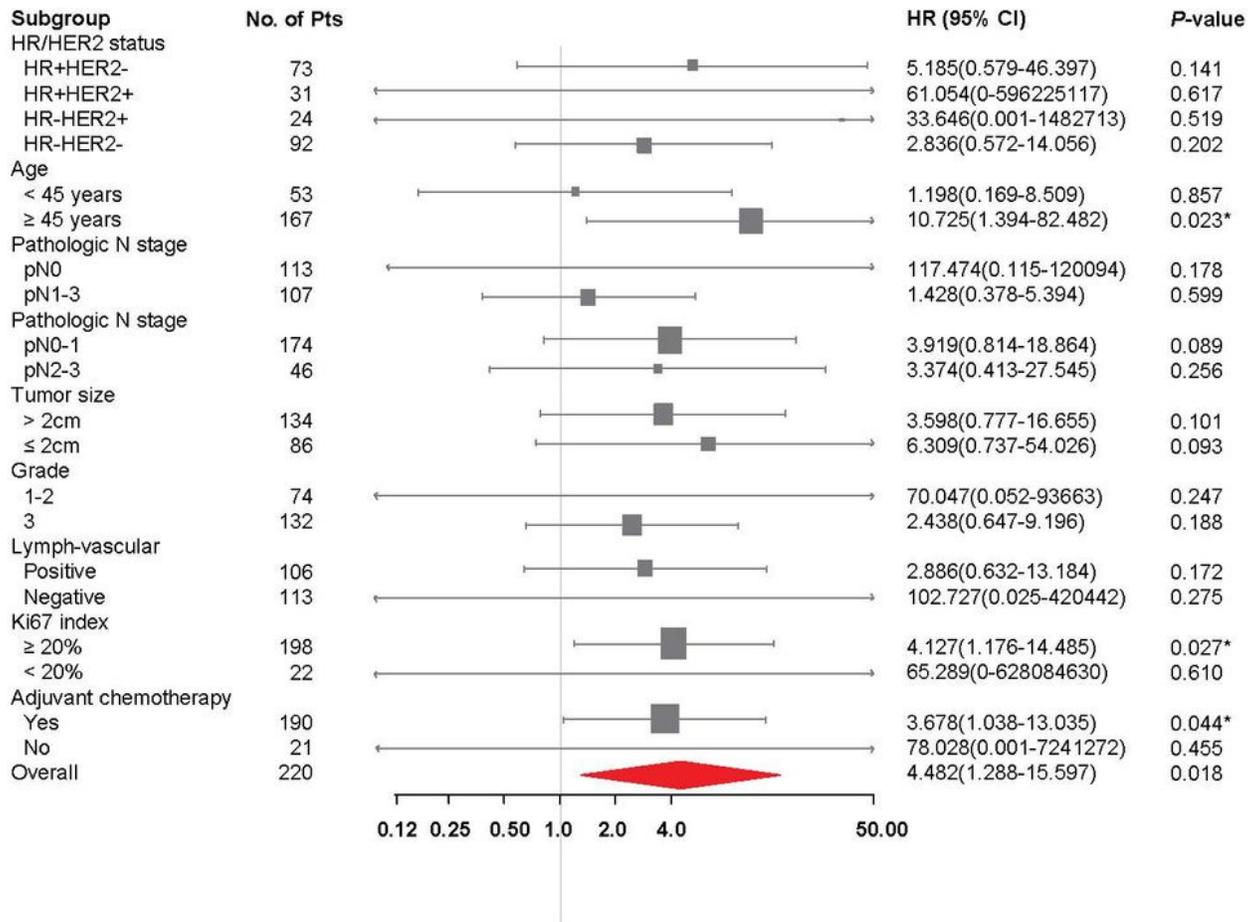


Figure 3

RFS hazard ratios in subgroups for comparison of ME1 expression level. Statistical analysis in each subgroup was performed by univariate cox proportional hazard model. X-axis shows HR and 95% CI in each subgroup. Arrows indicate that the limits of the confidence interval are not shown. Size of the box represents the relative number of patients in each group. Abbreviations: RFS, Recurrence free survival; ME1, Malic enzyme 1; Pts, Patients; HR, Hazard ratio; CI, Confidence interval. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

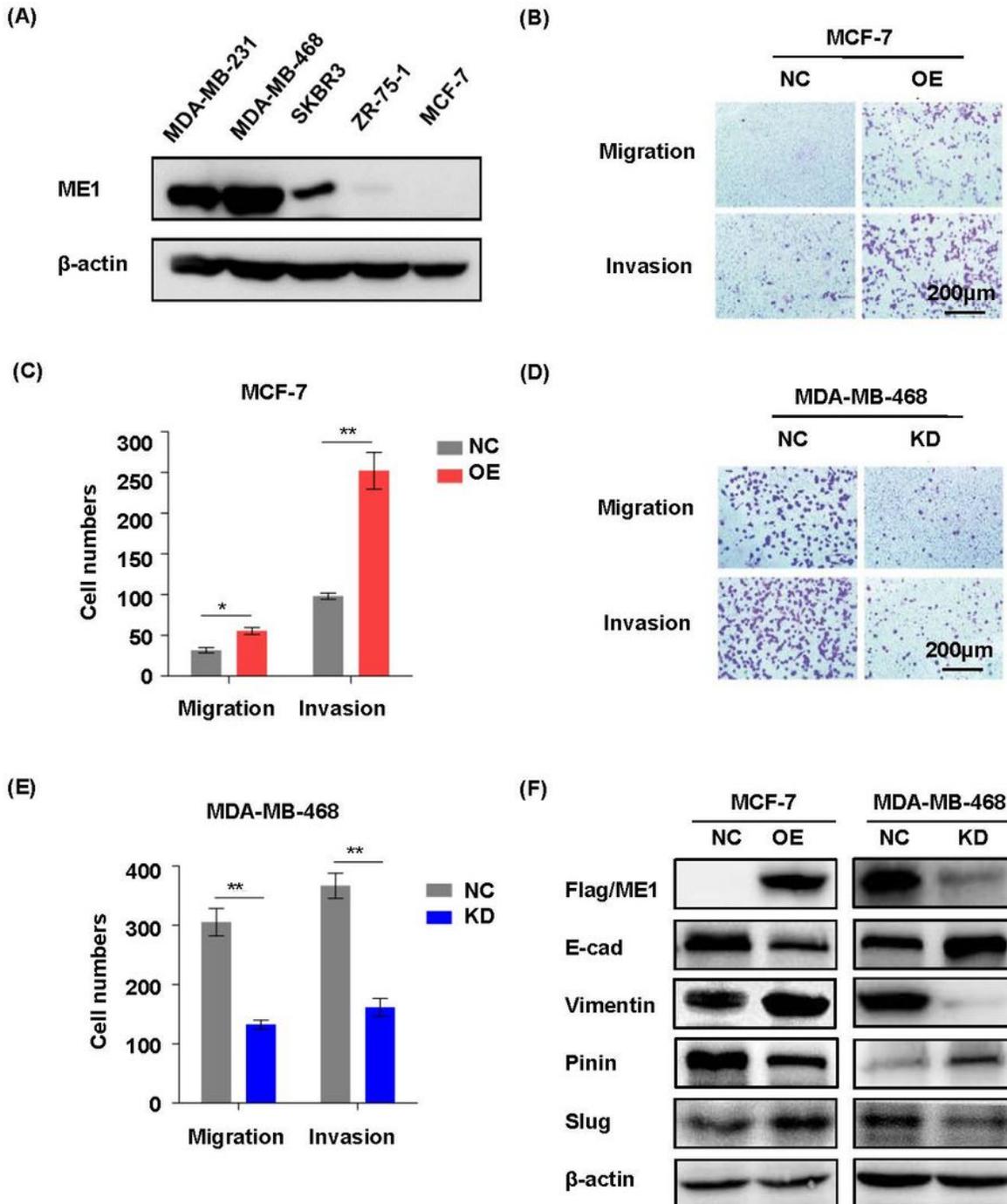


Figure 4

ME1 promoted motility and EMT in breast cancer cells. (A) Expression of ME1 in breast cancer cell lines with diverse malignancies examined by western blotting; (B-C) Transwell migration and invasion assays in MCF-7 cells (magnification, 100×); (D-E) Transwell migration and invasion assays in MDA-MB-468 cells (magnification, 100×); Data was represented as mean \pm SD of three independent experiments and analyzed with the Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: ME1, Malic enzyme 1; EMT, Epithelial-mesenchymal transition.

Figure 6

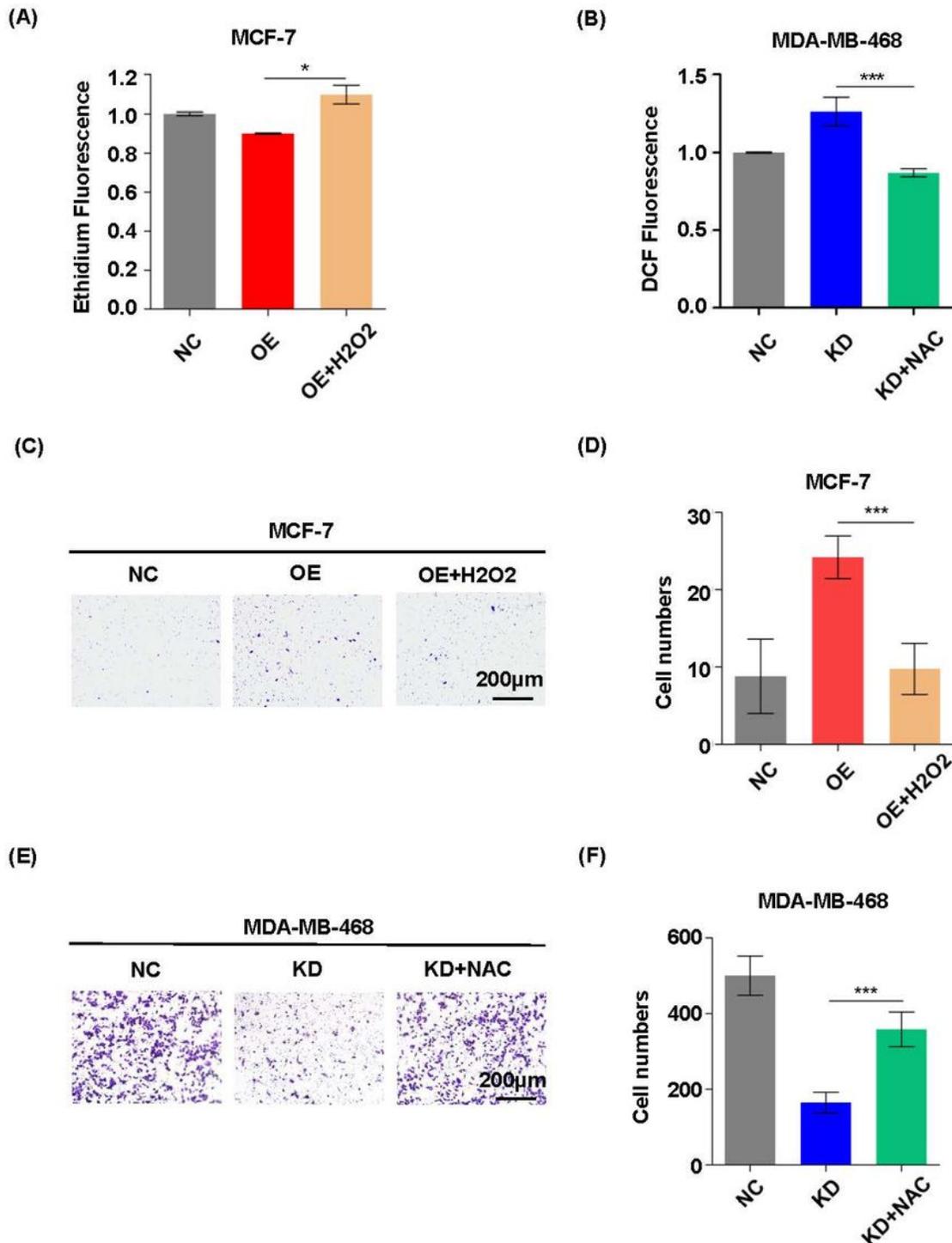


Figure 5

ME1 enhanced cell motility via ROS regulation in breast cancer cells. (A) Effect of H₂O₂ on cellular ROS level in MCF-7 cells; (B) Effect of NAC on cellular ROS level in MDA-MB-468 cells; (C-D) Transwell migration assays in MCF-7 cells pretreated with H₂O₂ (magnification, 100×); (E-F) Transwell migration assays in MDA-MB-468 cells pretreated with NAC (magnification, 100×); Data was represented as mean ± SD of three independent experiments and analyzed with the Student's t test. *P < 0.05, ** P < 0.01, ***P < 0.001. Abbreviations: ME1, Malic enzyme 1; ROS, Reactive oxygen species; H₂O₂, Hydrogen peroxide; NAC, N-acetyl cysteine.

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