Am J Transl Res 2020;12(3):773-786
www.ajtr.org /ISSN:1943-8141/AJTR0100702

Original Article

Adrenomedullin inhibits tumor metastasis and is associated with good prognosis in triple-negative breast cancer patients

Li-Li Liu[1,2*], Shi-Lu Chen[1,2*], Yu-Hua Huang[1,2*], Xia Yang[1,2], Chun-Hua Wang[1,2], Jie-Hua He[1,2], Jing-Ping Yun[1,2], Rong-Zhen Luo[1,2]

[1]Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, China; [2]Department of Pathology, Sun Yat-sen University Cancer Center, Guangzhou 510060, China. [*]Equal contributors and co-first authors.

Received July 31, 2019; Accepted February 3, 2020; Epub March 15, 2020; Published March 30, 2020

Abstract: Background: Cancer metastasis is the major reason for cancer-related deaths, but the mechanism of cancer metastasis still unclear. Adrenomedullin (ADM), a peptide hormone, functions as a local paracrine and autocrine mediator with multiple biological activities, such as angiogenesis, cell proliferation, and anti-inflammation. However, the expression and potential function of ADM in triple-negative breast cancer (TNBC) remain unclear. Methods: Real-time polymerase chain reaction and western blotting were performed to examine the expression of ADM in TNBC tissues and cell lines. A total of 458 TNBC tissue samples and adjacent nontumor tissue samples were detected by immunochemistry to determine the correlation between ADM expression and clinicopathological characteristics. We determined the role and mechanistic pathways of ADM in tumor metastasis in cell lines. Results: Our data showed that ADM expression was noticeably decreased in TNBC samples and cell lines. Low expression levels correlate with an increased risk of recurrence and metastasis. Furthermore, low ADM expression was associated with poor prognosis and was an independent marker for TNBC. In vitro, ADM may decrease cancer cell invasion, which is likely the result of its effect on the cancer cell epithelial-mesenchymal transition. Conclusions: Our findings suggest that ADM is a valuable biomarker for TNBC prognosis and an anti-metastasis candidate therapeutic target in triple-negative breast cancer.

Keywords: Adrenomedullin, triple-negative breast cancer patients, epithelial-mesenchymal transition

Introduction

Breast cancer is known as one of the most pervasive cancers [1]. According to global estimated cancer statistics, breast cancer is the second leading cause of cancer-related death among women [2]. Breast cancer is acknowledged as a heterogeneous disease with four major different molecular subtypes [3]. Among them, triple-negative breast cancer (TNBC) accounts for approximately 15% to 20% of all breast cancer and represents the most malignant breast cancer subtype [3, 4]. TNBC is defined as the absence of expression of estrogen receptor and progesterone receptor and the lack of amplification of human epidermal growth factor receptor 2 [4, 5]. It is well established that, as compared with the other subtypes of breast cancer, TNBC tends to exhibit aggressive, metastatic behavior; is associated with more frequent relapse; and has a worse prognosis [5]. Therefore, the need is urgent to identify more efficient therapeutic molecular targets and to develop novel prognostic biomarkers for TNBC patients.

Adrenomedullin (ADM) is a 52-amino-acid peptide initially isolated from human pheochromocytoma extracts by Kitamura et al. [6]. ADM has been identified as functioning as a multifunctional peptide [6]. Previous studies indicated that ADM has been implicated in a wide variety of cellular processes such as signaling transduction. ADM is now also known as a multifunctional peptide involved in angiogenesis, cell proliferation, and anti-inflammation [7-9]. ADM

also contributes to vascular regeneration and angiogenesis through activation of the PI3K/Akt, mitogen-activated protein kinase (MAPK), and endothelial nitric oxide synthase signaling pathways [10-12].

Recently, studies reported that ADM is widely expressed in various types of cancer, including osteosarcomas, pancreatic adenocarcinomas, prostate cancer, plexiform neurofibromas, and gastric adenocarcinomas [13-20]. Blocking ADM secretion from cancer cells or tumor-associated macrophages using a specific antibody or ADM antagonist inhibits tumor angiogenesis and growth. However, to date, the clinical significance and the role of ADM in TNBC remain unclear.

In this study, we intend to identify the role of ADM in TNBC. We provide here the first demonstration of the expression and clinical significance of ADM in TNBC. In the present study, we elucidate the expression character of ADM in breast cancer cell lines and a large cohort of clinical samples. The association between ADM expression and clinical pathological parameters was analyzed. Our data identified ADM as a novel prognostic biomarker and potential therapeutic target for BC patients.

Materials and methods

Patients, tissue specimens, and follow-up

A total of 458 human TNBC tissues and their corresponding nontumorous breast specimens (from January 2004 to December 2016) were collected from Sun Yat-sen University Cancer Center, Guangzhou, China. Those samples were analyzed along with clinical and pathological information obtained from patient records. Paired freshly resected TNBC tissues and corresponding adjacent nontumorous samples were collected for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot to explore ADM expression level. None of the patients had received radiotherapy or chemotherapy before surgery. This study was approved by the Institute Research Medical Ethics Committee. Since all samples were anonymously labeled, the written consents were waived.

Tissue microarray (TMA) construction and immunohistochemistry (IHC)

Tumorous tissue and adjacent nontumorous TNBC tissues were constructed for TMA. The TMA blocks were cut into 4-μm sections and subjected to IHC staining. The slides were dewaxed in xylene and alcohol and then treated with 3% hydrogen peroxide in methanol. After avidin-biotin blocking overnight at 4°C, the slides were incubated with ADM antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in phosphate-buffered saline three times, the slides were incubated with biotinylated goat anti-mouse antibodies. The slides were then stained with DAKO liquid 3,3'-diaminobenzidine tetrahydro-chloride (DAB) and finally counterstained with Mayer's hematoxylin.

Protein expression levels of ADM-stained TMA slides were observed under a microscope and assessed by two independent pathologists. The positively stained samples were scored as follows: 0, less than 5% positively stained cells; 1, 6% to 24% positively stained cells; 2, 25% to 49% positively stained cells; 3, 50% to 74% positively stained cells; 4, 75% to 100% positively stained cells. The intensity was scored as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was calculated by multiplying the percentage score by the staining intensity score. The median IHC score was chosen as the cutoff value for defining high and low ADM expression.

Cell lines and cell culture

Breast cancer cells were purchased from the Type Culture Collection Cell Bank, Chinese Academy of Science Committee (Shanghai, China) and routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco). All cells were maintained in a humidified incubator at 37°C and 5% CO_2. MCF-10A cells were non-neoplastic epithelia cells. ZR-75-1, SUM-159PT, T47D, MCF-7, MDA-NB-231, HCC1806 and HCC1937 were breast cancer (BC) cells. Among them, MDA-NB-231, SUM-159PT and HCC1937 were TNBC cells [21].

Western blot and antibodies

Total proteins from clinical samples and cultured cells were extracted and separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and then eletrophoretically transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking in 5% nonfat milk for 1 h at room temperature (20-25°C), the membranes were incubated with appropriately diluted primary antibodies overnight at 4°C. After washing with Tris-buffered saline with Tween, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:20,000 dilution for 1 h at room temperature. The membranes were visualized by the enhanced Phototope TM-HRP Detection Kit and exposed to the Bio-Rad processor. Antibodies used in this study were ADM (1:100, Santa Cruz, CA, USA) and β-actin (1:1000, Santa Cruz, CA, USA).

QRT-PCR and primer sequences

Total RNA from clinical samples and cultured cells was extracted by Trizol reagent (BIOO Scientific Co., USA). Reverse transcription and SYBR green-based real-time PCR were conducted. The primers were designed as follows: ADM forward: 5'-TGTCGTCATTGGACACGTAGA-3' and reverse: 5'-ACGCTCAGCTTTCAGTTTATCC-3'; 18S forward: 5'-TGAGAAACGGCTACCACATCC-3' and reverse: 5'-ACCAGACTTGCCCTCCAATG-3'.

Cell migration and invasion assays

Cells were resuspended in 200 μL of serum-free medium and then placed in the upper compartment of a Transwell chamber (Corning; 24-well insert, pore size: 8 μm). The lower chamber was filled with 15% fetal bovine serum as a chemoattractant and incubated for 48 h for the migration assay and 72 h for the invasion assay. For the invasion assay, the inserts were previously coated with extracellular matrix gel (BD Biosciences, Sparks, MD, USA). At the end of the experiments, the cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet and counted. Five visual fields were randomly chosen, and the number of cells was counted under a microscope.

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software (SPSS, Chicago, IL, USA). Student's t-test was used to determine the significance of differences in ADM expression levels. Data reported in bar or column charts are depicted as mean ± SEM. Pearson's χ^2 test or Fisher's exact test was performed to analyze the correlation between ADM expression level and the clinicopathological parameters. Survival was analyzed by Kaplan-Meier method, while factors associated with survival were identified by the Cox proportional hazard regression model. Differences were considered significant when *P* values were less than 0.05.

Results

ADM expression was downregulated in BC cell lines and fresh tissue samples

To identify the expression of ADM in BC, cell lines and TNBC fresh tissue samples were detected by qRT-PCR and western blot. The results showed that ADM mRNA expression was downregulated in the BC cell lines compared with that in the immortalized breast cell lines MCF-10A, in agreement with The Cancer Genome Atlas (TCGA) data and Curtis data (**Figure 1A** and **1E**). Similarly, ADM showed the same decreased protein expression levels in BC cell lines (**Figure 1B**). To further evaluate the expression of ADM, 18 pairs of BC fresh tissues resected from patients with primary BC were collected. ADM mRNA expression was markedly decreased in the BC samples compared with that in the matched nontumor tissues (**Figure 1C**). Consistently, the protein level of ADM was markedly decreased in 83.3% (5/6) of the TNBC fresh tissues compared with the corresponding adjacent nontumorous paired samples (**Figure 1D**).

Downregulated expression of ADM in TNBC detected by IHC

To further confirm the expression profile of ADM in TNBC, 458 archived paraffin-embedded TNBC samples were collected and constructed into a TMA cohort along with clinical and pathological data. The representative IHC images of ADM expression in the tumor were observed with high, medium, and low staining (**Figure 2A**). ADM was primarily located in the

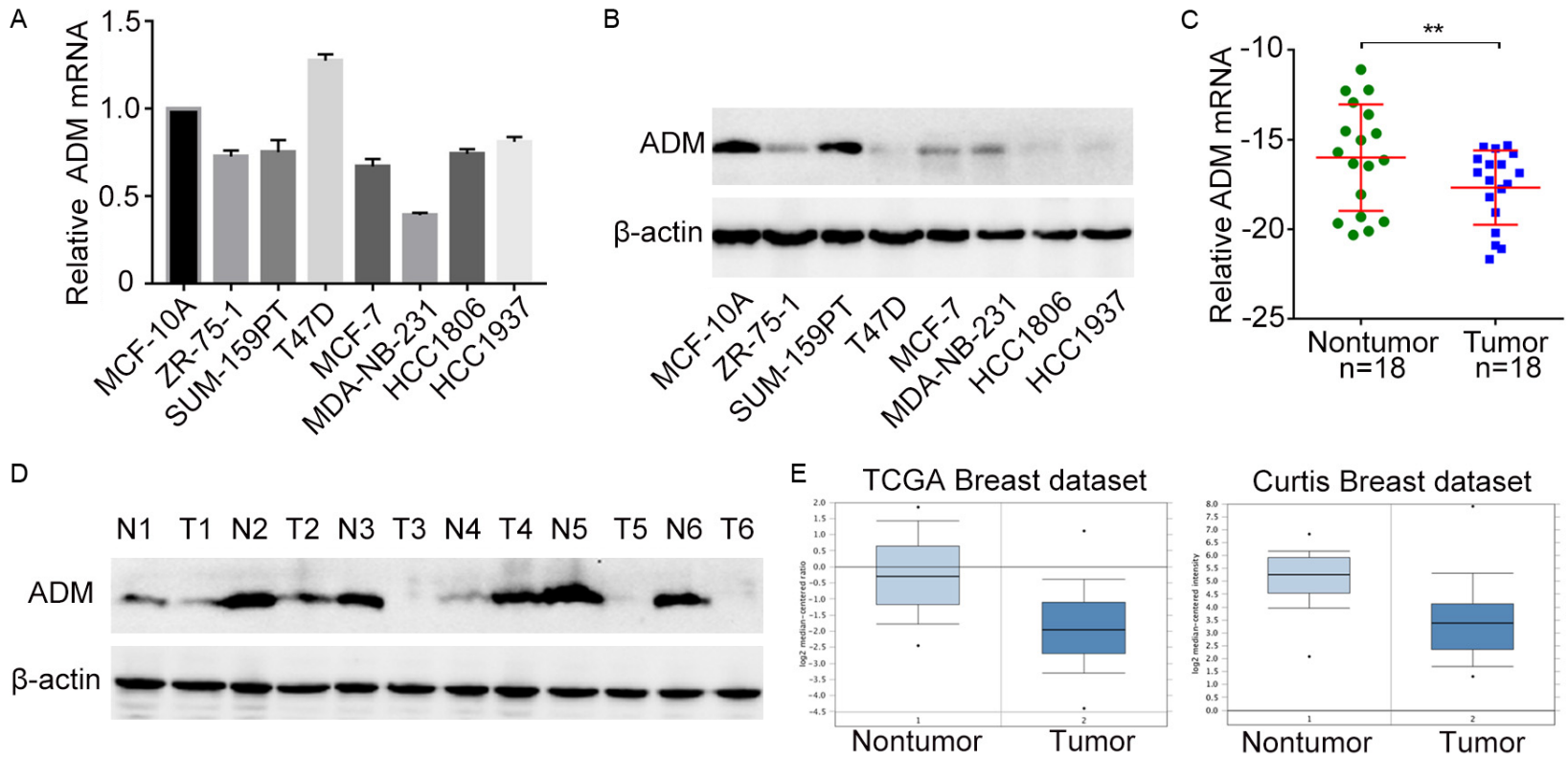

Figure 1. ADM expression was decreased in breast cancer (BC) cell lines and fresh tissue samples. A. The mRNA expression levels of ADM in BC cell lines and immortalized breast cell lines examined by qRT-PCR. ADM mRNA expression was downregulated in the BC cell lines compared with that in the immortalized breast cell lines MCF-10A. The assay was conducted three times independently. B. The protein expression levels of ADM in breast cancer and immortalized breast cell lines detected by western blot and decreased ADM expression levels were observed in BC cell lines. C. Expression profile of ADM mRNA in 18 pairs of BC and corresponding adjacent breast tissues; ADM mRNA expression was markedly decreased in the BC samples compared with that in the matched nontumor tissues. D. ADM protein expression were decreased in 5/6 paired TNBC and adjacent nontumorous tissues. E. Expression profile of ADM mRNA in BC and corresponding adjacent breast tissues from TCGA and the Curtis breast data set.

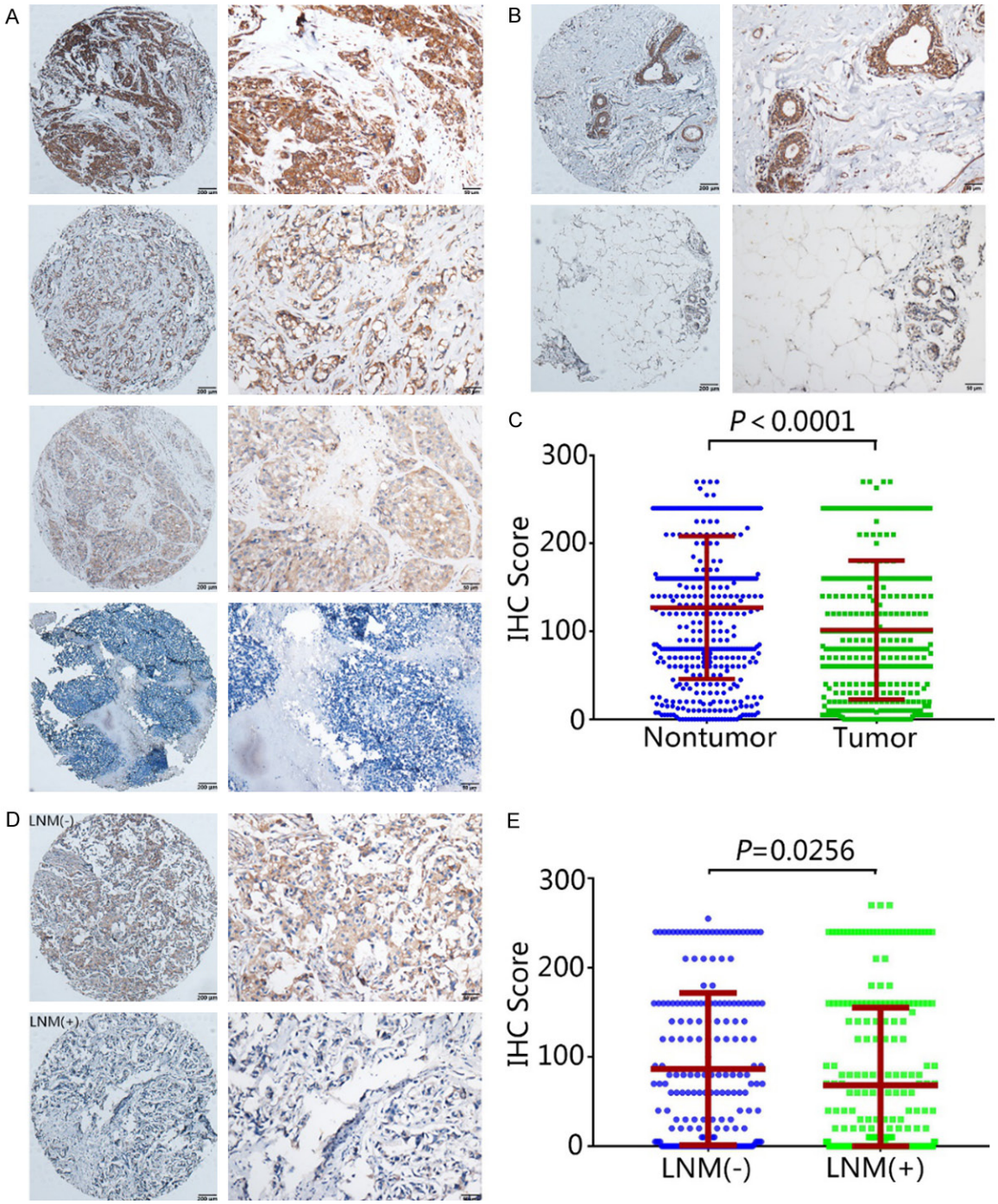

Figure 2. Down expression of ADM in TNBC detected by IHC. A. Representative images of IHC staining for ADM expression in a TMA cohort. Representative images of strong (top), moderate (middle), weak (middle), and negative (bottom) intensity staining for tumor tissues are shown. B. Representative IHC images of positive (top) and negative (bottom) nontumor tissues are presented. C. The IHC scores of the TMA cohort, including 458 TNBC patients. ADM expression in TNBC was remarkably lower than that in paired-matched nontumorous tissues. D. ADM expression in TNBC with lymph node metastasis (LNM) venus TNBC without LNM cases analyzed by IHC. Representative photomicrographs are shown for the LNM negative (LNM-) and positive (LNM+) lesions. E. Higher ADM immunoreactivity was detected in TNBC without lymphatic metastases than in TNBC with lymphatic metastases.

cytoplasm. ADM expression in TNBC was remarkably lower than that in paired-matched nontumorous tissues (**Figure 2B** and **2C**). Moreover, we found that higher ADM immuno-

Table 1. Correlation of clinicopathological parameters and ADM expression (n = 458)

Variable	ADM expression			*P* value[a]
	All cases	Low expression	High expression	
Age (years)[b]				0.723
< 49	250	151 (60.4%)	99 (39.6%)	
≥ 49	208	129 (62.0%)	79 (39.0%)	
Menopause				0.720
No	280	173 (61.8%)	107 (38.2%)	
Yes	178	107 (60.1%)	71 (39.9%)	
Diabetes				0.774
No	429	263 (61.3%)	166 (38.7%)	
Yes	29	17 (58.6%)	12 (41.4%)	
Hypertension				0.800
No	396	243 (61.4%)	153 (38.6%)	
Yes	62	37 (59.7%)	25 (40.3%)	
Pathological type				0.806
Ductal carciconoma	444	271 (61.0%)	173 (39.0%)	
Other	14	9 (64.3%)	5 (35.7%)	
Tumor size (cm)[c]				0.940
< 3.2	302	185 (61.3%)	117 (38.7%)	
≥ 3.2	156	95 (60.9%)	61 (39.1%)	
Grade				0.994
I-II	162	99 (61.1%)	63 (38.9%)	
III	296	181 (61.1%)	115 (38.9%)	
Vascular invasion				0.934
No	356	218 (61.2%)	138 (38.8%)	
Yes	102	62 (60.8%)	40 (39.2%)	
Nerve invasion				0.250
No	434	268 (61.8%)	166 (38.2%)	
Yes	24	12 (50.0%)	12 (50.0%)	
pT stage				0.660
pTis-pT2	416	253 (60.8%)	163 (39.2%)	
pT3-pT4	42	27 (64.3%)	15 (35.7%)	
pN stage				0.050
pN0-pN2	421	252 (59.9%)	169 40.1%)	
pN3	37	28 (75.7%)	9 (24.3%)	
M stage				0.525
M0	442	269 (60.9%)	173 (39.1%)	
M1	16	11 (68.8%)	5 (31.3%)	
TNM stage				0.826
0-II	350	213 (60.9%)	137 (39.1%)	
III-IV	108	67 (62.0%)	41 (38.0%)	
Ki-67				0.551
< 14%	73	47 (64.4%)	26 (35.6%)	
≥ 14%	385	233 (60.5%)	152 (39.5%)	

[a]Chi-square test; [b]Median age; [c]Median tumor size.

reactivity was detected in 204 TNBC without lymphatic metastases than in 254 TNBC with lymphatic metastases (**Figure 2D** and **2E**). Taken together, our data indicated that ADM is decreased in TNBC compared with nontumor tissues and is even lower in TNBC with lymphatic metastases.

Association of ADM expression and TNBC patient survival

To determine the clinical significance of ADM in TNBC, the correlation between ADM expression and the clinicopathological variables of TNBC patients was analyzed. According to the mean IHC staining score of ADM, the TNBC cases were divided into two groups: high ADM expression and low expression. High expression of ADM was observed in 38.8% (178/458) of the cases. Statistical analysis indicated that high ADM expression was associated with pN stage (P = 0.05, **Table 1**). Next, we determined the prognostic implication of ADM in TNBC patients. Kaplan-Meier analysis showed that patients with high ADM expression had longer overall survival compared with the low ADM group (mean, 202.1 months vs. 157.8 months, respectively, $P < 0.001$; **Figure 3A**). Low ADM expression was also associated with poor disease-free survival (DFS; mean, 117.9 months vs. 122.1 months, respectively, $P < 0.037$; **Figure 3B**) and the tendency for relapse in TNBC patients (**Figure 3C**). Stratified survival analysis further confirmed the prognostic value of ADM. Stratification analyses further demonstrated that ADM expression was associated with a series of pathological parameters related to overall survival (**Figure 4** and Supplementary Figure 1). Therefore, our data demonstrated that ADM can predict the clinical outcome of patients with TNBC.

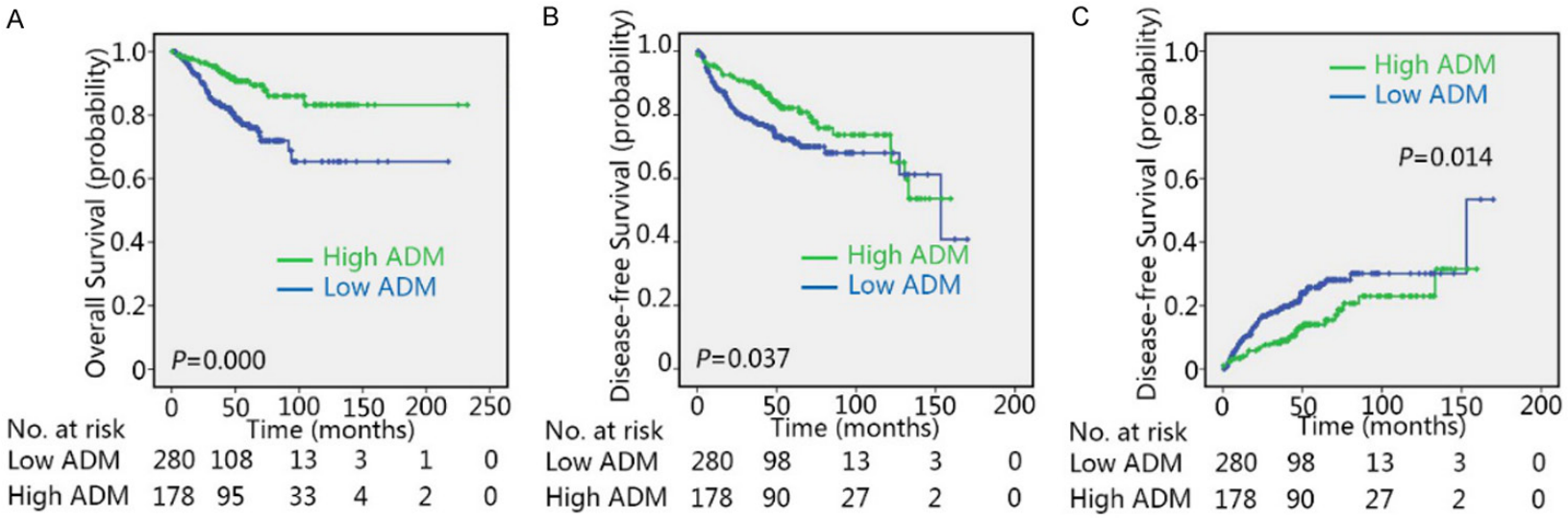

Figure 3. Association of ADM expression and TNBC patient survival. A. The correlation of ADM expression and overall survival was determined in a TMA cohort including 458 patients by Kaplan-Meier analysis. Results showed that patients with high ADM expression had longer overall survival compared with the low ADM group (mean, 202.1 months vs. 157.8 months, respectively, $P < 0.001$). B. Disease-free survival of the same ADM TMA cohort. Low ADM expression was also associated with poor disease-free survival (mean, 117.9 months vs. 122.1 months, respectively, $P < 0.037$). C. Low ADM expression was also associated with tendency of relapse in TNBC patients. The life table is shown in each graph below.

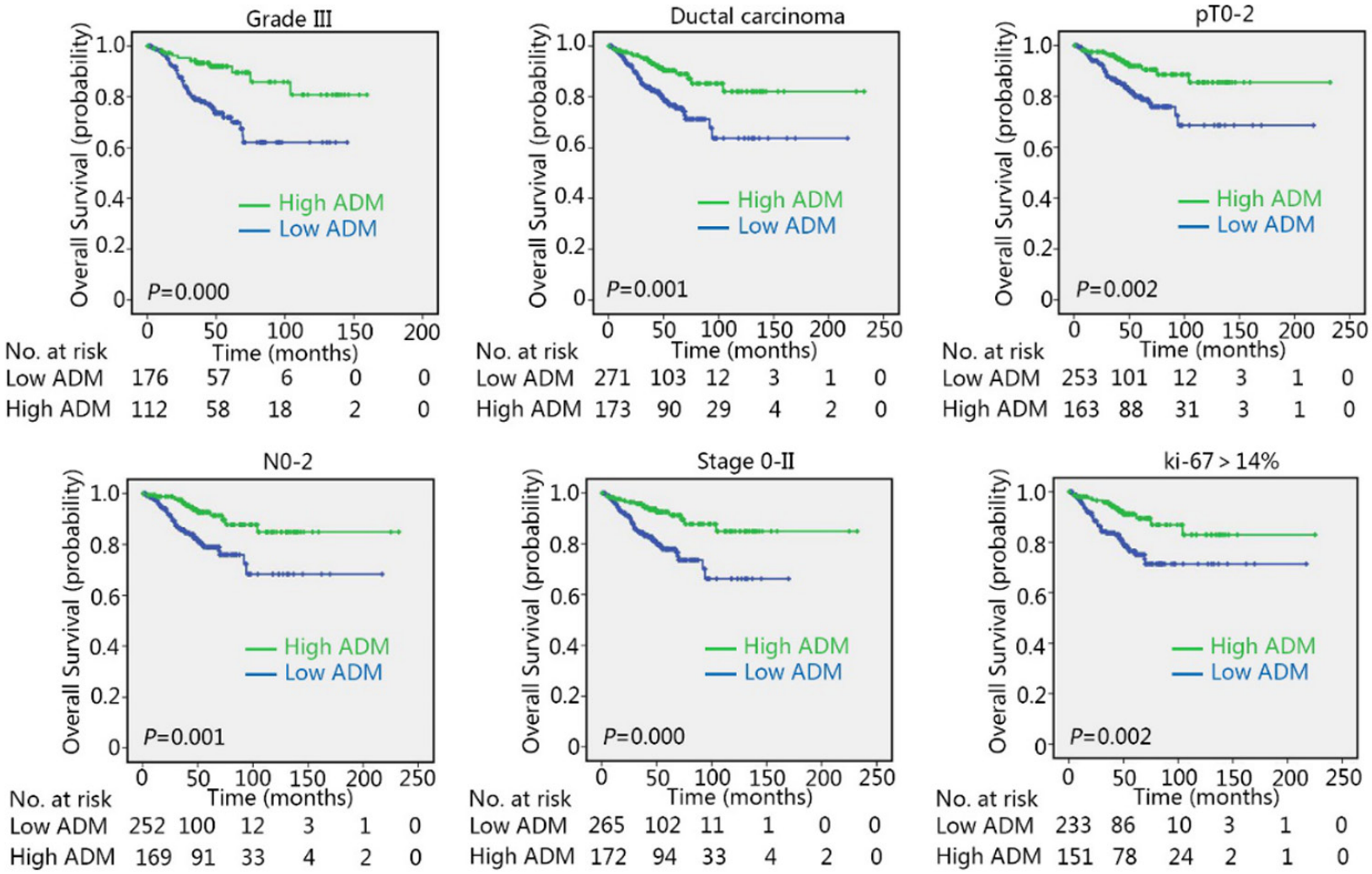

Figure 4. Stratified analysis of ADM expression related to overall survival. The correlation of ADM expression and overall survival in the indicated groups. Patients with high ADM expression had longer overall survival compared with the low ADM group among TNBC patients with Grade III, with Ductal carcinoma patients, with pT0-2, with N0-2, with stage 0-II and with ki-67 > 14%.

Table 2. Univariate and multivariate analyses of clinicopathological and ADM expression for overall survival in overall cohort (n = 458)

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Overall survival				
Age (< 49 vs. ≥ 49 years)	1.803 (1.134-2.866)	0.013		
Menopause (no vs. yes)	2.079 (1.309-3.302)	0.002	2.600 (1.209-5.591)	0.014
Diabetes (no vs. yes)	1.184 (0.513-2.733)	0.692		
Hypertension (no vs. yes)	1.390 (0.776-2.492)	0.268		
Pathological type (ductal carciconoma vs. other)	0.307 (0.043-2.220)	0.307		
Tumor size (< 3.2 vs. ≥ 3.2 cm)	2.028 (1.281-3.211)	0.003		
Grade (I-II vs. III)	1.811 (1.072-3.059)	0.026	1.718 (1.005-2.936)	0.048
Vascular invasion (no vs. yes)	1.708 (1.018-2.867)	0.043		
Nerve invasion (no vs. yes)	2.041 (0.815-5.109)	0.128		
pT stage (0-2 vs. 3-4)	3.726 (2.137-6.499)	0.000		
pN stage (0-2 vs. 3)	4.273 (2.417-7.554)	0.000		
M stage	4.918 (2.351-10.289)	0.000		
TNM (I-II vs. III-IV)	4.611 (2.911-7.304)	0.000	3.170 (1.698-5.920)	0.000
Ki-67 (< 14% vs. ≥ 14%)	0.952 (0.530 -1.709)	0.869		
ADM expression (low vs. high)	0.398 (0.233-0.680)	0.001	0.432 (0.248-0.752)	0.003

HR, hazard ratio; CI, confidence interval.

Univariate and multivariate analyses of prognostic variables in TNBC

To further evaluate the prognostic value of ADM in TNBC, Cox regression analysis was conducted. ADM expression was identified as a prognostic factor (hazard ratio [HR] = 0.398, 95% confidence interval [CI] 0.233-0.680, *P* = 0.001). After adjusting for the prognostic factors, multivariate Cox regression analysis revealed ADM was an independent factor for better overall survival (HR = 0.432, 95% CI 0.248-0.752, *P* = 0.003), as shown in **Table 2**. However, although ADM was closely related to DFS in survival analysis and univariate analysis, there was no significant difference observed between ADM expression and DFS in multivariate analysis (*P* = 0.088), as shown in **Table 3**. In conclusion, our data indicated ADM as an independent prognostic marker of overall survival for TNBC patients.

ADM inhibits cell-cell invasion and migration in BC

Due to the links between ADM expression and patient characteristics, we next investigate the effect of ADM in BC cell function on cell mobility. We observed that inhibition of ADM expression can significantly inhibit cell invasion, and overexpression of ADM increased cell invasion in the MDA-MB-231 and MDA-MB-468 cell line (**Figure 5**). Transwell assays showed that the overexpression of ADM was reduced, whereas the knockdown of ADM reduced the ability of cell invasion and migration in BC cells (**Figure 5A**, **5B**). Wound-healing assays further demonstrated that ADM-depleted cells filled the wound faster than cells with ADM expression. By contrast, overexpression of ADM hindered cell mobility (**Figure 5C**, **5D**). These data indicate that ADM promotes cancer metastasis in BC cells, but it may not be absolutely required for cell growth or proliferation.

We also investigated whether there is an effect of ADM in BC cell proliferation. However, we found that changes in ADM expression did not affect cell growth. In addition, we carried out a clone formation assay and found that changes in ADM expression did not affect cell clone formation abilities (Supplementary Figure 2).

The mechanism that ADM affects metastasis may be through the epithelial-mesenchymal transition (EMT)

We next went on to investigate the role of ADM in BC cell invasion. The AKT phosphorylation signaling pathway and its downstream EMT sig-

Table 3. Univariate and multivariate analyses of clinicopathological and ADM expression for Disease-free survival in overall cohort (n = 458)

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Overall survival				
Age (< 49 vs. ≥ 49 years)	1.131 (0.770-1.660)	0.531		
Menopause (no vs. yes)	1.453 (0.990-2.134)	0.056		
Diabetes (no vs. yes)	0.3184 (0.122-1.213)	0.103		
Hypertension (no vs. yes)	1.334 (0.811-2.195)	0.256		
Pathological type (ductal carciconoma vs. other)	0.764 (0.242-2.412)	0.646		
Tumor size (< 3.2 vs. ≥ 3.2 cm)	1.370 (0.925-2.030)	0.116		
Grade (I-II vs. III)	1.087 (0.729-1.619)	0.684		
Vascular invasion (no vs. yes)	2.053 (1.348-3.216)	0.001	1.495 (0.942-2.373)	0.088
Nerve invasion (no vs. yes)	3.286 (1.743-6.195)	0.000	3.450 (1.770-6.726)	0.000
pT stage (0-2 vs. 3-4)	3.681 (2.295-5.902)	0.000	2.230 (1.288-3.861)	0.004
pN stage (0-2 vs. 3)	2.892 (1.670-5.008)	0.000	1.269 (0.661-2.434)	0.474
M stage	4.486 (2.382-8.449)	0.000	2.028 (0.992-4.149)	0.053
TNM (I-II vs. III-IV)	3.359 (2.279-4.950)	0.000	1.956 (1.155-3.313)	0.013
Ki-67 (< 14% vs. ≥ 14%)	1.026 (0.616 -1.708)	0.921		
ADM expression (low vs. high)	0.651 (0.434-0.976)	0.038	0.695 (0.457-1.056)	0.088

HR, hazard ratio; CI, confidence interval.

naling pathway plays an essential role in BC metastasis. To investigate the mechanism of ADM in BC metastasis, we examined the effect of ADM on the AKT pathway and EMT in BC cells. When overexpressed ADM, the level of AKT phosphorylation was remarkedly decreased, whereas when we knocked down ADM expression, the AKT phosphorylation level significantly increased (**Figure 6A**, **6B**). The EMT marker of E-cadherin was increased, and the mesenchymal cell marker N-cadherin was decreased with ADM overexpression. Moreover, knockdown of ADM expression promoted expression of the mesenchymal cell marker N-cadherin and decreased the expression of epithelial cell marker E-cadherin (**Figure 6C**, **6D**). These data indicate that ADM mediates BC cell migration and invasion by the cell EMT.

To further elucidates the mechanism of the role of ADM in tumor cells, we performed a potential protein-protein interaction analysis of ADM (Supplementary Figure 3). We found that decreased ADM significantly activates the hypoxia-inducible factor 1A (HIF1A) signaling pathway.

Discussion

TNBC bears the worst prognosis of any breast cancer, because it is resistant to most conventional therapies [4, 22]. Metastasis is the main cause of death in women with TNBC [4, 22]. To improve the outcome of TNBC patients, the potential underlying molecular mechanism should be elucidated to aid in the discovery of more reliable treatment targets and prognostic biomarkers. Here, we show that ADM expression is decreased in TNBC and strongly correlated with favorable overall survival and DFS. Multivariate analysis indicated ADM as an independent prognostic predictor. In addition, we found that ADM promotes TNBC cell metastasis by the AKT and EMT pathway. To our knowledge, this is the first study to unveil the clinical significance and potential molecular mechanism of ADM in TNBC.

Dysregulation of ADM has been reported in human cancers [13]. However, the expression of ADM in various tumors is not consistent. ADM was shown to be overexpressed in gastric tumors, demonstrating oncogenic properties [18, 19]. Moreover, overexpression of ADM was found to be associated with poorer prognosis in gastric cancer [18, 19]. However, the expression profile and clinical significance of ADM in breast cancer have rarely been demonstrated. In this study, ADM expression was found to be decreased in a cohort of 458 patients with TNBC. TNBC patients with high ADM expression had a longer life, both in overall survival and

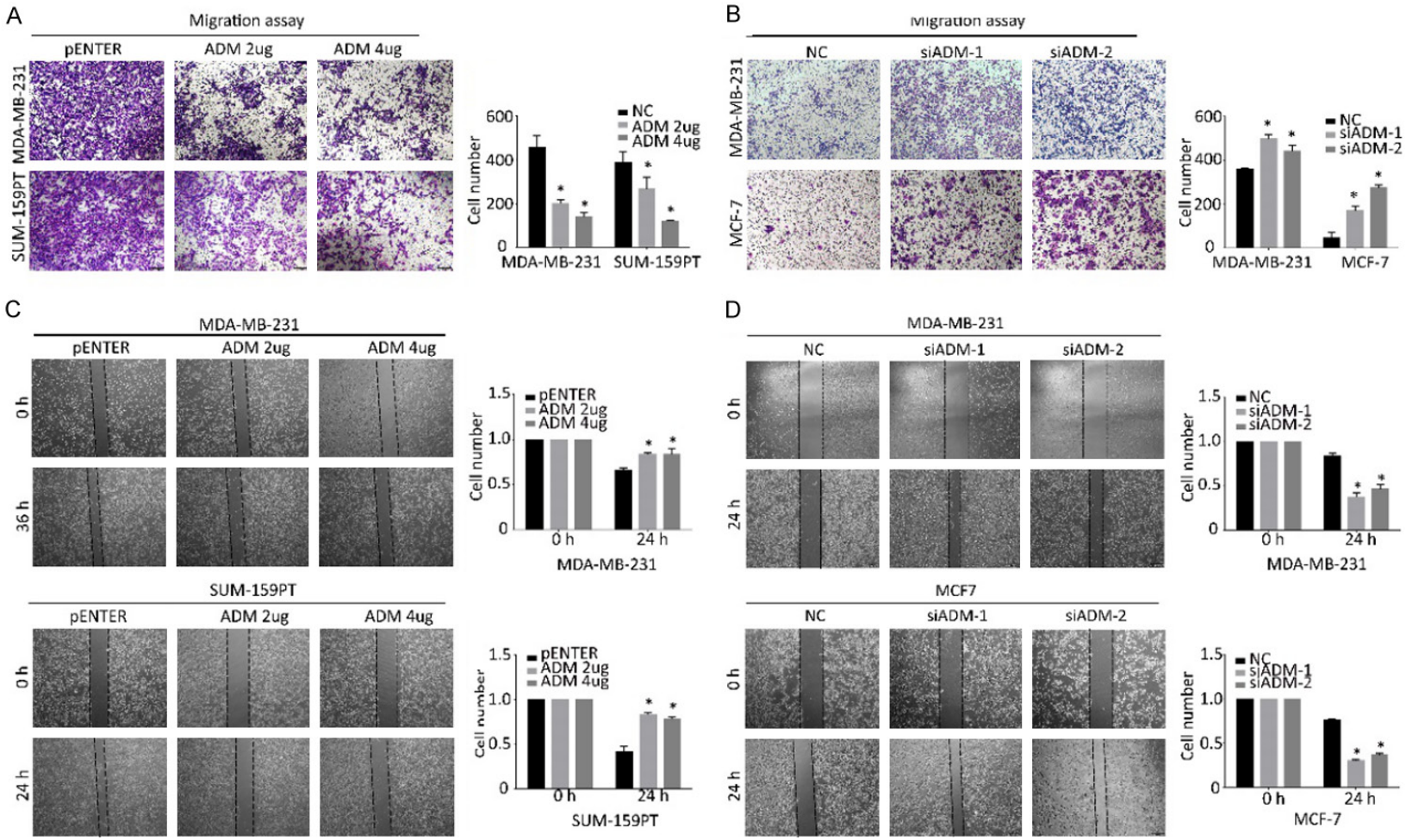

Figure 5. The effects of ADM expression on TNBC cell migration. We use transwell assays to detect the invasion and migration abilities in cells with ADM overexpression or depletion. In transwell assay, (A) Overexpression of ADM expression can significantly inhibit cell invasion while (B) knockdown of ADM increased cell invasion in the MDA-MB-231 and MDA-MB-468 cell line. In wound-healing assays, (C) overexpression of ADM hindered cell mobility, (D) ADM-depleted cells filled the wound faster than cells with ADM expression. Representative images and quantitative data of three randomly selected fields are shown. Wound-healing assays demonstrated cell movement capacity in ADM-expressing knockdown (C) of AIM2 knockdown (D) cells. Quantitative data are presented as the mean SD. $*P < 0.05$; $**P < 0.01$.

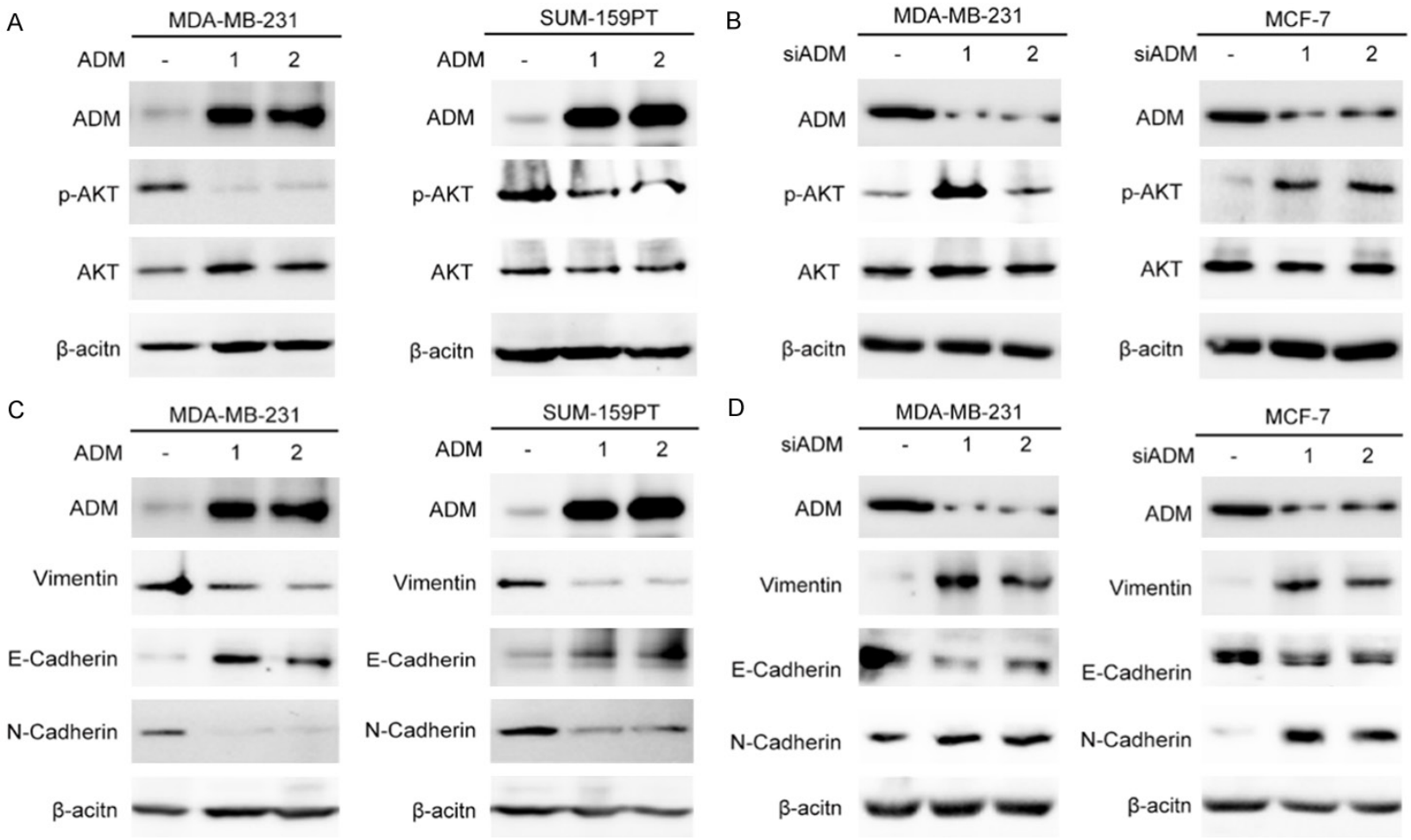

Figure 6. The mechanism of ADM affecting TNBC cell metastasis may be through EMT. (A) Overexpression ADM reduces the level of AKT phosphorylation, (B) whereas when we knocked down ADM expression, the AKT phosphorylation level significantly increased. (C) The EMT marker of E-cadherin was increased, and the mesenchymal cell marker N-cadherin was decreased with ADM overexpression (D). Knock down of ADM expression promoted expression of the mesenchymal cell marker N-cadherin and decreased the expression of epithelial cell marker E-cadherin.

DFS, compared with the low-expression group. These data suggest that ADM might be a novel prognostic biomarker for TNBC patients. Interestingly, using a specific antibody for ADM, we showed that ADM mainly localized in the cytoplasm of TNBC cells. In addition, we showed that ADM inhibits the cell EMT and may play a direct role in influencing cancer spread.

A previous study reported that ADM is an important protein in metabolism and tumor physiology, and metabolism is also closely related to tumor physiology [23-25]. For example, ADM could stimulate adipose tissue lipolysis in an autocrine manner by activating ERK1/2 and p38 MAPK pathways [26]. In our study, we found that ADM could also activate the AKT signaling pathway. The AKT pathway is a very important signaling pathway [27, 28]. Important cellular events are regulated downstream of the AKT pathway, including cell proliferation and cell migration [27-29]. In the present study, we found that ADM can activate the AKT signaling pathway of TNBC cells and stimulate the EMT processes to promote cell motility. However, ADM did not affect the cell cycle of TNBC cells. This may illustrate a new mechanism for elucidating the molecular role played by ADM in TNBC. In our *in vitro* experiments, we used not only two TNBC cell lines, but also a BC cell line. We found that down-regulated ADM in those three cell lines promoted the EMT process of tumor cells and therefore promoted the cell motility. The results imply that AMD may not only plays a role in inhibiting metastasis in TNBC, but also may have a similar effect in BC. However, it still needs further research to confirm.

In summary, our data demonstrated increased ADM expression in TNBC cell lines and clinical samples. A decreased ADM expression level predicted unfavorable overall survival. Collectively, our study suggests ADM as promising new biomarker for the prognosis of patients with TNBC.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81602135, 81872012, 81872266) and the National Natural Science Foundation of Guangdong Province (No. 2016A030310215). The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number RDDB2020000652.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Rong-Zhen Luo, Department of Pathology, Sun Yat-sen University Cancer Center, Guangzhou 510060, Guangdong, China. Tel: +8620-87342272; Fax: +8620-87342272; E-mail: luorzh@sysucc.org.cn

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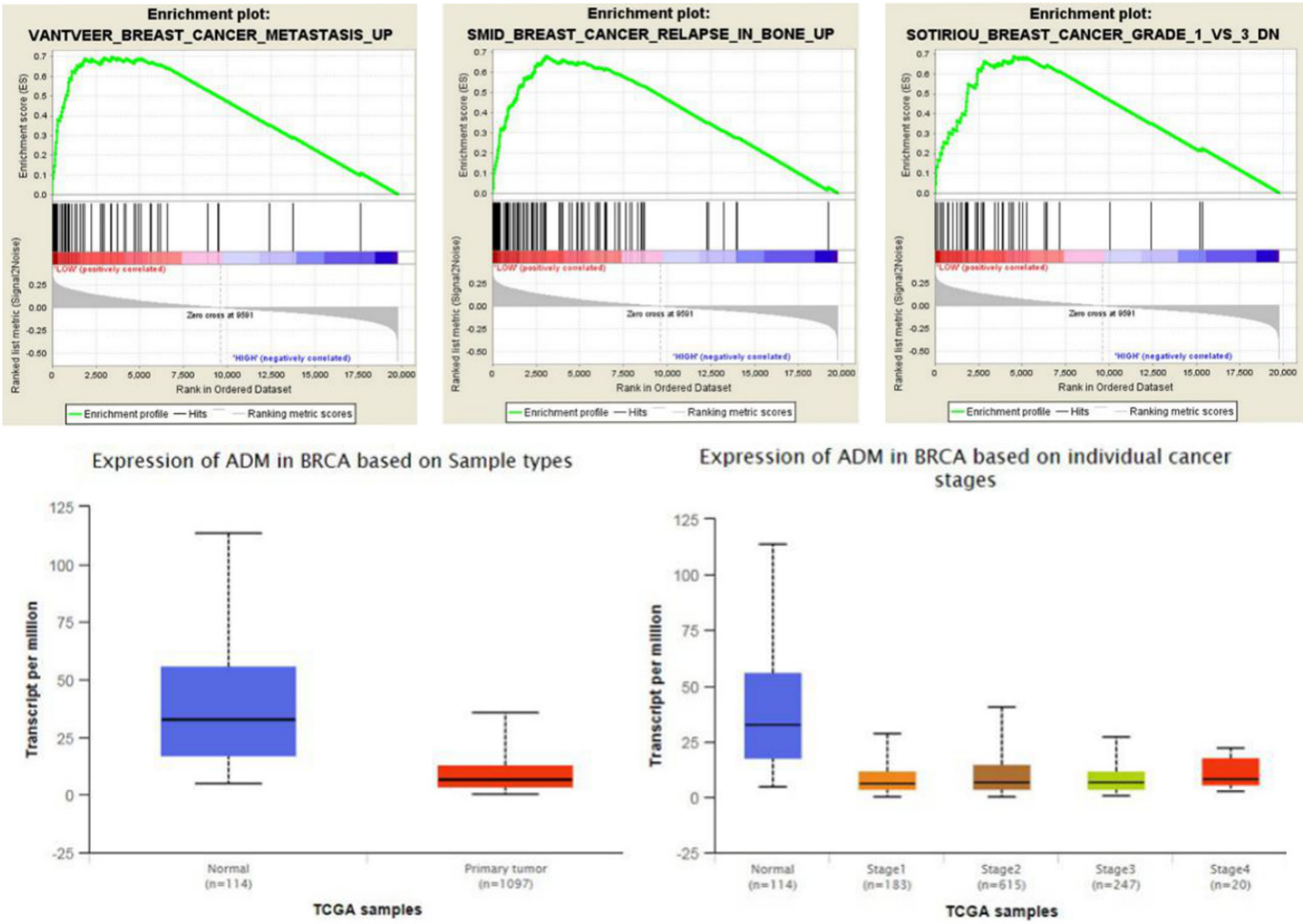
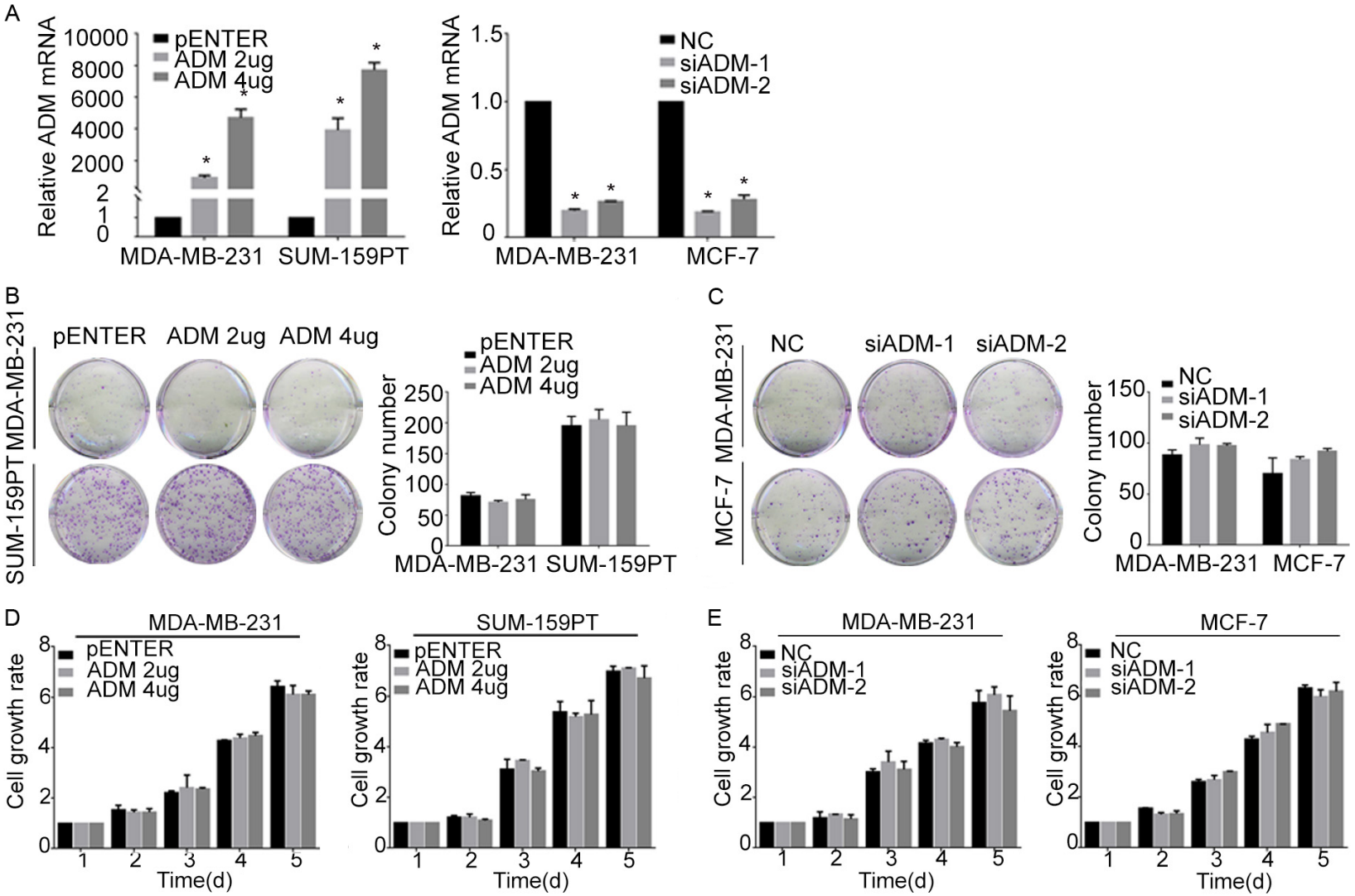
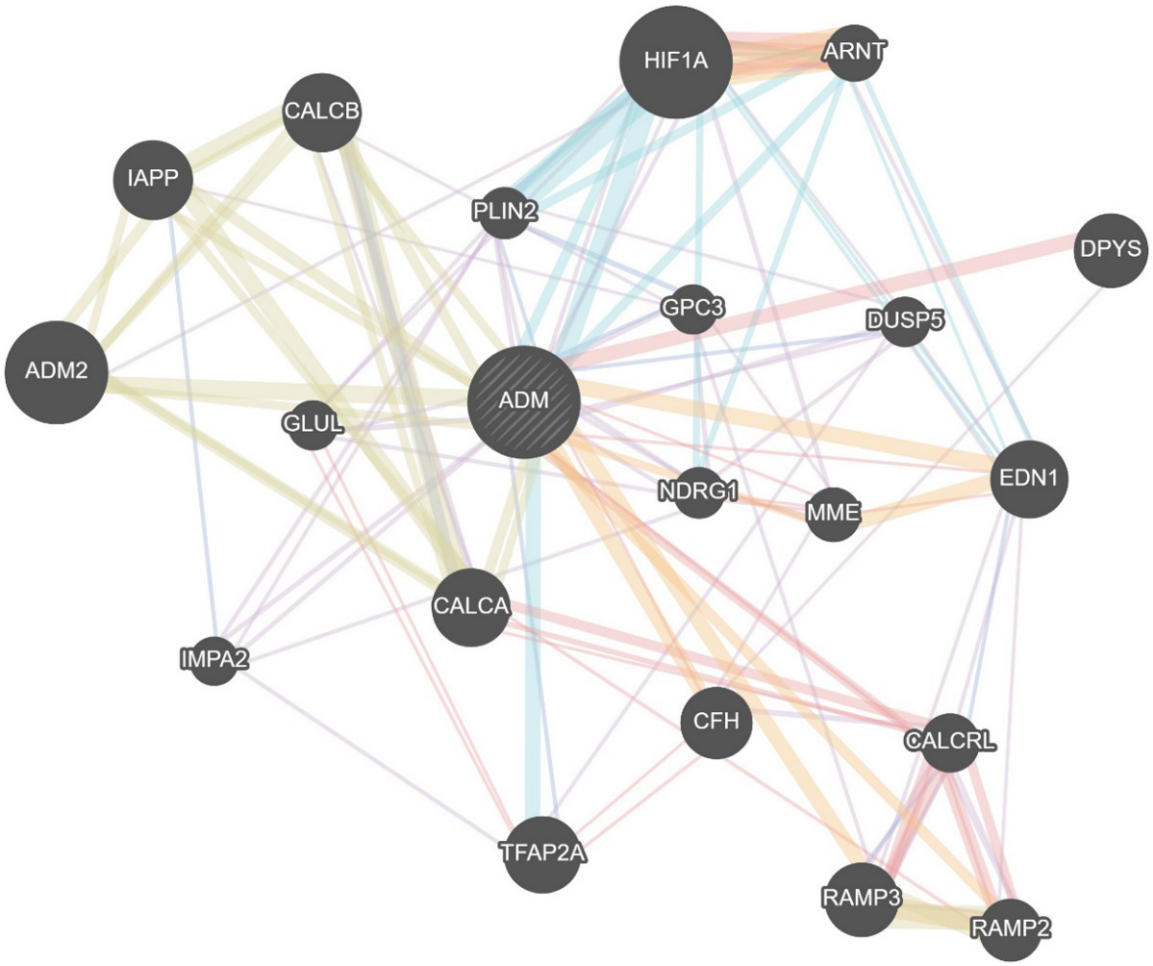


Supplementary Figure 1. ADM enrichment and expression level in available online database. We used an available online database to evaluate the ADM expression profile, and the results showed that ADM was decreased in breast cancer cells. ADM expression was associated with TNBC with different stage.



Supplementary Figure 2. ADM expression did not affect cell proliferation. (A) ADM expression was confirmed in breast cancer cells using qRT-PCR. (B) Clone formation was detected after cells were transfected with ADM overexpression vector or (C) knocked down with AMD siRNA. (D) MTT assay was conducted after cells were transfected with ADM overexpression vector or (E) knocked down with ADM siRNA.



Supplementary Figure 3. We performed a potential protein-protein interaction analysis of ADM. We found that decreased ADM significantly activates the hypoxia-inducible factor 1A signaling pathway.