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Overexpressed DAAM1 correlates with metastasis and predicts poor prognosis in breast cancer

Jie Mei[a,b,1], Bujie Xu[a,1], Leiyu Hao[a], Zhuang Xiao[a], Yan Liu[a], Ting Yan[c], Yichao Zhu[a,d,*]

[a] Department of Physiology, Nanjing Medical University, Nanjing 211166, China
[b] Department of Oncology, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi 214023, China
[c] Safety Assessment and Research Center for Drug, Pesticide and Veterinary Drug of Jiangsu Province, Nanjing Medical University, Nanjing 211166, China
[d] State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 211166, China

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ABSTRACT

Recent studies have reported that dishevelled-associated activator of morphogenesis 1 (DAAM1) is remarkably essential for mediating cell migration and invasion in breast cancer (BrCa). Nonetheless, the definite expression profile of DAAM1 in BrCa patients and the impact on metastasis of BrCa *in vivo* have not been explored up to now. The differential expression of DAAM1 in BrCa and adjacent tissues was assessed *via* immunohistochemistry (IHC) staining. The metastatic capacities of BrCa SUM-1315 cells were examined in BALB/c nude mice. Besides, the prognostic values of *DAAM1* mRNA in BrCa were explored based on Kaplan-Meier (KM) plotter. The expression of DAAM1 protein was notably overexpressed in BrCa tissues compared with that in paired normal breast tissues. The high expression of DAAM1 in BrCa tissues was significantly associated with lymph-node metastasis. Furthermore, DAAM1 overexpression promoted the invasive capacity of BrCa cells and stimulated lung metastatic extent *in vivo*. We also found that overexpressed *DAAM1* mRNA was significantly associated with poor relapse-free survival (RFS), overall survival (OS), distance-metastasis-free survival (DMFS), and post-progression survival (PPS). Our findings reveal that DAAM1 might be a novel therapeutic target to manage the deteriorated metastasis of BrCa and identified DAAM1 as a promising biomarker for unfavorable prognosis in BrCa patients.

1. Introduction

Breast cancer (BrCa) has the highest morbidity among female malignancies worldwide and it causes an increasing number of cancer-related deaths [1]. The American Cancer Society (ACS) predicts that there will be more than 270,000 new BrCa cases and approximately 40,000 deaths in the United States in 2019 [2]. Although treatments for BrCa have achieved great progress, advanced BrCa with lymph-node and/or distant metastasis is still lack of effective therapies to limit the malignant progression [3]. Thus, it is still significant to explore the underlying molecular mechanisms of BrCa metastasis and potential prognostic biomarkers of BrCa.

Dishevelled-associated activator of morphogenesis 1 (DAAM1) is a formin protein that mediates cytoskeletal rearrangement *via* regulating Wnt/PCP (planar cell polarity) signaling pathway [4,5]. DAAM1 largely enhances the elongation of actin filament and is participated in multiple actin-dependent cellular behaviors [6–8]. Dysregulated expression of DAAM1 often occurs in various tumors [9–12]. Our previous studies have demonstrated that DAAM1 transduces the signal of Wnt5a and type-IV collagen and activates the downstream effector RhoA, thereby promoting RhoA-mediated microfilament formation in BrCa cells [13,14]. However, the expression of DAAM1 in BrCa specimens and its function on metastasis have not been evaluated *in vivo*.

The Kaplan-Meier plotter (KM plotter, http://kmplot.com) is an online database to evaluate the prognostic effect of genes expression on survival in designate cancers. KM plotter contains gene expression profiles and survival data from the Gene Expression Omnibus (GEO), the European Genomephenome Archive (EGA) and the Cancer Genome Atlas (TCGA) database [15]. The dominating purpose of the platform is to assess prognosis-related biomarkers based on meta-analysis, and increasing amounts of prognostic biomarkers have been identified on this platform [16–19].

* Corresponding author at: Department of Physiology, Nanjing Medical University, 101 Longmian Av., Nanjing 211166, China.
E-mail addresses: meijie199621@163.com (J. Mei), bujiexu192@163.com (B. Xu), leiyuhao316@163.com (L. Hao), xiaozhuang_325@163.com (Z. Xiao), kldliuyan@163.com (Y. Liu), yanting@njmu.edu.cn (T. Yan), zhuyichao@njmu.edu.cn (Y. Zhu).
[1] Equal contribution.

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Table 1
The basic clinicopathological features in two patients' cohorts.

Characteristics	Total cases	Cases (%)	
		Outdo Biotech cohort	Recruited cohort
Tumor size			
≤2cm	25	5 (16.7%)	20 (43.5%)
> 2cm	50	25 (83.3%)	25 (54.3%)
unknown	1	0 (0%)	1 (2.2%)
Lymph-node metastasis			
N0	52	16 (53.3%)	36 (78.3%)
N1-N3	24	14 (46.7%)	10 (21.7%)
Distant metastasis			
M0	75	30 (100%)	45 (97.8%)
M1	1	0 (0%)	1 (2.2%)
ER status			
Negative	31	17 (56.7%)	14 (30.4%)
Positive	45	13 (43.3%)	32 (69.6%)
PR status			
Negative	39	20 (66.7%)	19 (41.3%)
Positive	37	10 (33.3%)	27 (58.7%)
HER2 status			
Negative	45	20 (66.7%)	25 (54.3%)
Positive	31	10 (33.3%)	21 (45.7%)
Intrinsic subtypes			
TNBC	15	8 (26.7%)	7 (15.2%)
Others	61	22 (73.3%)	39 (84.8%)

In the current study, we examined the expression of DAAM1 protein in BrCa and adjacent tissues and the metastatic capacities of DAAM1-ovexpressed BrCa cells. Furthermore, we assessed the prognostic effects of DAAM1 mRNA on patients' survival based on BrCa cohorts in KM plotter. Given together, we verified the role of DAAM1 on BrCa and suggested DAAM1 as a metastatic promotor and prognostic biomarker in BrCa.

2. Materials and methods

2.1. Clinical samples

A total of 76 BrCa samples were contained in this research. Forty-six BrCa patients were recruited by the Jiangsu Province Hospital and Jiangsu Cancer Hospital from 2015 to 2018. Besides, a tissue microarray slide (HBre-Duc060CS-02) containing 30 BrCa and adjacent tissues was purchased from Outdo Biotech (Shanghai, China). The basic clinicopathological features were described in Table 1. All cases had been diagnosed with BrCa by pathologists according to hematoxylin-eosin (HE) staining. Clinical staging was determined based on American Joint Committee on Cancer (AJCC) Cancer Staging Manual 7th classification criteria. Ethical approval for the research was obtained from the Clinical Research Ethics Committee, Nanjing Medical University.

2.2. Cell culture and transfection

SUM-1315 BrCa cell line was kindly gifted from Dr. Tiansong Xia (Nanjing Medical University). SUM-1315 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone) at 37 °C with 5% CO_2. The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and the details of plasmids were described in our previous publication [13]. The cells stably-transfected with Mock and DAAM1 were used for further animal assay.

2.3. Quantitative real-time PCR

Total RNA of SUM-1315 cells was extracted using Trizol reagent. Then, we performed qRT-PCR to measure *DAAM1* mRNA expression in SUM-1315 cells. Primers used for DAAM1 amplification were GAPDH: 5'-TGAACGGGAAGCTCACTGG-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense); DAAM1: 5'-AAATTGAAACGGAATCGCAAAC-3' (sense) and 5'-GCAAGGCAGTGTAATGAAACG-3' (antisense). SYBR Green (SYBR® Premix Ex Taq™ II, TaKaRa, Dalian, China) was used to label the amplified genes. The $2^{-\Delta\Delta Ct}$ method was used for DAAM1 expression analysis.

2.4. Western blotting

SUM-1315 cells were placed into 35-mmdishes (6×10^5 cells/dish) for extracting total proteins. Seventy-two hours later, SUM-1315 cells were harvested and homogenized with lysis buffer. Cellular lysates were separated by denatured 10% SDS-PAGE. DAAM1 and GAPDH primary antibodies (Proteintech, Wuhan, China) were used. GAPDH was used to normalize DAAM1 protein levels.

2.5. Mice xenograft models

Four-week-old female BALB/c nude mice weighing 18–22 g were purchased from Department of Laboratory Animal Centre (Nanjing Medical University). A total of 20 mice were involved in this research, which were randomly divided into two groups including 5 mice in control group and 5 in experimental group for different experiments. For tumor growth and metastasis, stable DAAM1-overexpressed SUM-1315 cells and control cells were inoculated in the both anterior limbs of nude mice subcutaneously (1×10^6 cells in 100 μL PBS) and injected into mice *via* tail veins (2×10^6 cells in 100 μL PBS), respectively. Body weights of each mouse were assessed once per week. After 4 weeks, all mice were terminated by animal euthanasia. Then, subcutaneous tumors and lung tissues were collected for growth and metastasis evaluation and standard histopathologic study. Ethical approval for the animal experiment was obtained from the Animal Ethics Committee, Nanjing Medical University.

2.6. Immunohistochemistry (IHC) staining

Immunohistochemistry staining was directly performed on the tissue slides. The primary antibodies used were as follows: anti-DAAM1 (1:100 dilution, Cat. 14876-1-AP, ProteinTech, Wuhan, China). Antibody staining was visualized with DAB and hematoxylin counterstain (ZSGB-Bio). The percentage of positively stained cells was scored on a scale of 0 to 4 as follows: 0: < 1%, 1: 1–25%, 2: 25–50%, 3: 50–75% and 4: > 75%. The staining intensity was scored from 0 to 3 as follows: 0: negative, 1: weak, 2: moderate, and 3: strong. The immunoreactivity score (IRS) for each case was generated by multipling percentages of positive cells and staining intensities. Immunostained sections were photographed using a microscope (Olympus Corporation, Tokyo, Japan).

2.7. Kaplan-Meier plotter analysis

The prognostic values of *DAAM1* (Affymetrix ID: 216060_s_at) mRNA in BrCa were analyzed using the KM Plotter, an online database containing gene expression profiles and survival data of BrCa patients. According to the expression of *DAAM1* mRNA, the cases in the database were ranked from low expression to high expression. The bottom 50% were divided into the low expression group and the top 50% belonged to the high expression group. All cohorts were compared with KM survival plots. Hazard ratio (HR), 95% confidence interval (95%CI), and log rank *P* value were calculated.

2.8. Statistical analysis

All statistical analyses were performed using SPSS 25.0 and Graphpad prism 6.0 softwares. Most statistical comparison among

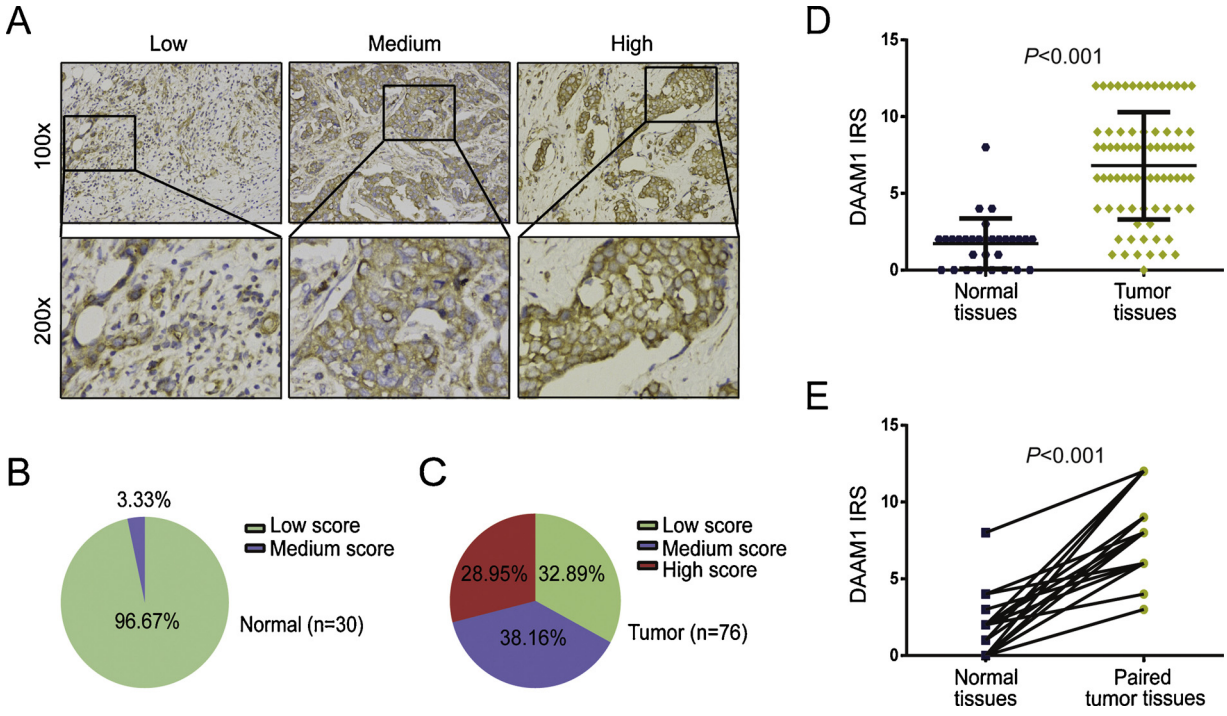

Fig. 1. DAAM1 protein expression in BrCa and adjacent tissues.
(A) The representative microphotographs revealing low, medium, and high DAAM1 staining intensities based on IHC staining. Brown, DAAM1; Blue, haematoxylin. Bar = 100 μm. (B, C) DAAM1 protein expression intensity proportion of BrCa tissues and adjacent tissues. Low expression: IRS ≤ 4; Medium expression: 4 < IRS ≤ 8; High expression: IRS > 8. (D, E) The expression of DAAM1 protein in BrCa tissues and adjacent tissues. (D) Compared with normal breast tissues (n = 30), expression of DAAM1 was significantly increased in BrCa tissues (n = 76). (E) A notable increase of DAAM1 expression was observed in BrCa tissues (n = 30) compared with adjacent tissues (n = 30).

Table 2
Associations between DAAM1 expression levels and clinicopathological features of BrCa.

Characteristics	Cases	DAAM1 IRS[a]			χ2	*P* value
		Low	Medium	High		
Tumor size						
≤2cm	25	11	10	4	3.624	0.163
> 2cm	50	14	18	18		
unknown	1					
Lymph-node metastasis						
N0	52	23	16	13	9.675	**0.008**
N1-N3	24	2	13	9		
Distant metastasis						
M0	75	25	29	21	2.145	0.289[b]
M1	1	0	0	1		
ER status						
Negative	31	10	13	8	0.381	0.827
Positive	45	15	16	14		
PR status						
Negative	39	11	18	10	2.180	0.336
Positive	37	14	11	12		
HER2 status						
Negative	45	16	16	13	0.433	0.805
Positive	31	9	13	9		
Intrinsic subtypes						
TNBC	15	5	7	3	0.873	0.646
Others	61	20	22	19		

[a] Low expression: IRS ≤ 4; Medium expression: 4 < IRS ≤ 8; High expression: IRS > 8.
[b] *P* value for Fisher's exact test.

different groups was analyzed by Student's *t*-test. The associations between DAAM1 expression status and clinicopathological features were assessed using Pearson's chi-squared test or Fisher's exact test. KM survival plots were generated with survival curves compared by log-rank test, but Tarone-Ware test was used when crossover between the groups was observed in survival plots. For all analyses, differences were considered statistically significant if *P* values were less than 0.05.

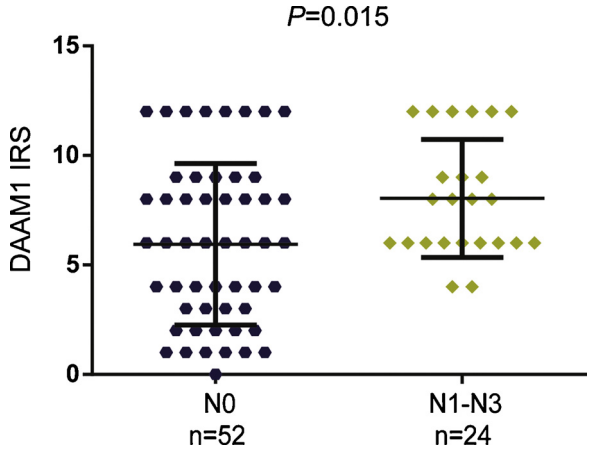

Fig. 2. DAAM1 protein expression according to the status of lymph-node metastasis in BrCa.
The expression of DAAM1 in BrCa tissues was detected using IHC staining. Compared with non-lymph-node metastatic tissues, DAAM1 protein expression was significantly increased in lymph-node metastatic tissues. N0, without lymph-node metastasis. N1-N3, with lymph-node metastasis.

3. Results

3.1. DAAM1 was overexpressed in BrCa tissues

We performed IHC staining on 76 BrCa tissue sections to measure DAAM1 expression difference (Fig. 1A). After the analysis of 30 BrCa

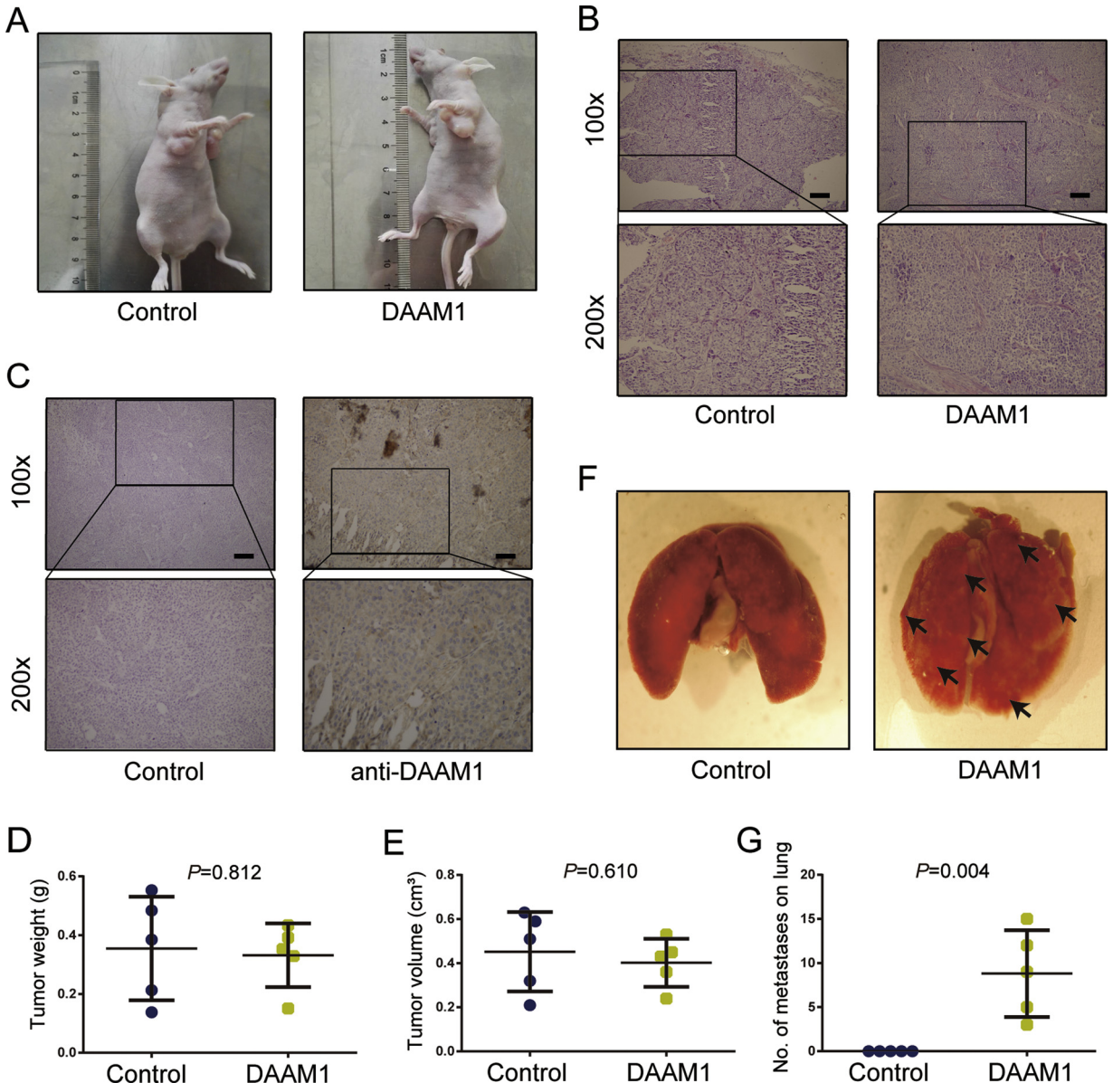

Fig. 3. Effect of DAAM1 overexpression on BrCa metastases in mice xenografts.
(A) Stable DAAM1-overexpressed SUM-1315 cells and control cells were injected into both anterior limbs of nude mice. The representative photographs of xenografts. (B) Representative images of HE stained sections of the xenografts. Bar = 100 μm. (C) DAAM1 expression in SUM-1315 cells was confirmed by immunohistochemistry. Bar = 100 μm. DAAM1 was highly expressed in SUM-1315/DAAM1-overexpressed tumor. (D, E) The tumor masses were carefully dissected and the tumor weight and volume (the average of two sides) were measured after the mice were killed. No significant difference of the tumor weight and volume between the two groups was exhibited. (F) Mice lungs were dissected for metastasis evaluation. The arrows showed lung metastases nodes. (G) Overexpressed DAAM1 notably promotes the lung metastasis compared with control group.

cases, we found that the sections moderately and highly-expressed DAAM1 accounted for a majority of tumor tissues, while most normal sections exhibited lowly-expressed DAAM1 (Fig. 1B and C). Moreover, the protein expression of DAAM1 in the BrCa tissues was notably up-regulated than that in adjacent tissues ($P < 0.001$, Fig. 1D, E). These findings along with our previous results at the cellular level [13,14,20,21] imply DAAM1 functions as an oncogene in BrCa.

3.2. Overexpressed DAAM1 was associated with lymph-node metastasis in BrCa

When we used $\chi 2$ test to analyze the association with DAAM1 expression status and clinicopathological features of total 76 BrCa cases, we found that high expression of DAAM1 was significantly associated with lymph-node metastasis of BrCa ($P = 0.008$, Table 2). DAAM1 expression was not associated with other patients' features, including tumor size, distant metastasis, ER status, PR status, HER2 status, and intrinsic subtypes ($P > 0.05$, Table 2). Furthermore, we compared DAAM1 expression in lymph-node metastatic BrCa tissues and non-lymph-node metastatic BrCa tissues. DAAM1 expression was notably upregulated in lymph-node metastatic tissues ($P = 0.015$, Fig. 2). Overall, the analysis of DAAM1 protein level in clinical samples verified the critical role of DAAM1 in lymph-node metastasis of BrCa.

3.3. DAAM1 overexpression stimulates BrCa metastasis in vivo

We further examine the influence of DAAM1 overexpression on cell proliferative and migratory capacity in xenograft model. At first, we measured the DAAM1 mRNA and protein level in SUM-1315 cells transfected with DAAM1 to guarantee the transfection efficiency

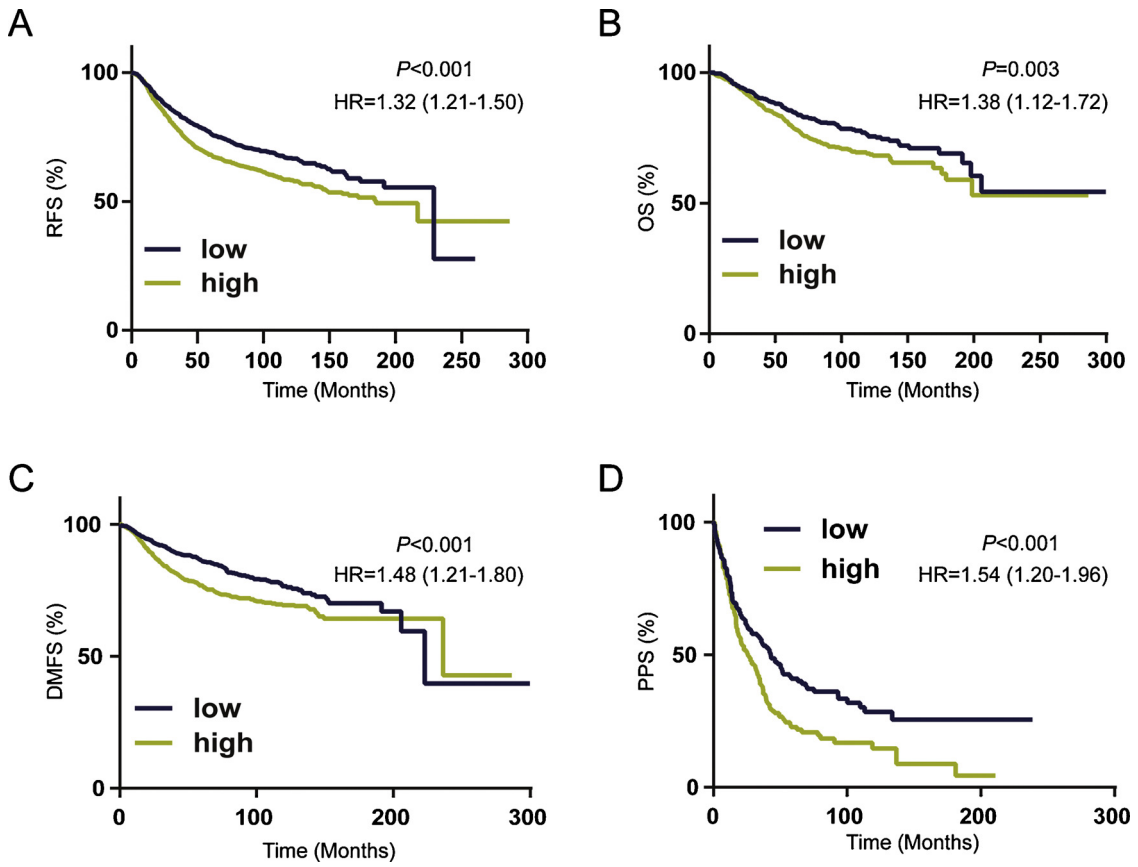

Fig. 4. The prognostic values of *DAAM1* mRNA expression in BrCa patients.
Affymetrix ID was valid: 216060_s_at (*DAAM1*). (A) RFS curve was plotted for all BrCa patients (n = 3951). RFS of patients with high expression of *DAAM1* mRNA was significantly worse than that of patients with low expression. (B) OS curve was plotted for all BrCa patients (n = 1402). OS of patients with high expression of *DAAM1* mRNA was significantly worse than that of patients with low expression. (C) DMFS curve was plotted for all BrCa patients (n = 1746). DMFS of patients with high expression of *DAAM1* mRNA was significantly worse than that of patients with low expression. (D) PPS curve was plotted for all BrCa patients (n = 414). PPS of patients with high expression of *DAAM1* mRNA was significantly worse than that of patients with low expression.

(Figure S1A, S1B). Next, SUM-1315 cells were inoculated in both anterior limbs of nude mice subcutaneously to assay tumor cells proliferation capacity and injected into mice *via* tail veins to evaluate BrCa cells arrest in lung during blood flow (Fig. 3A and F). After the mice euthanasia, we performed histologic detection of the xenograft tumors to confirm whether subcutaneous tumors were BrCa cells mass (Fig. 3B). IHC staining showed upregulated DAAM1 expression in tumor tissues in the DAAM1-overexpressed group (Fig. 3C). All of the results verified that the xenograft model was successfully constructed. Next, we found the tumor volume and weight were shown non-significant difference in DAAM1-overexpressed group and control group (Fig. 3D and E). As we expected, the lung metastasis was notably upgraded when compared with control group ($P = 0.004$, Fig. 3G). Taken together, these results suggested that DAAM1 has no obvious effect on tumors growth, but largely stimulates BrCa cells lung metastatic extent *in vivo*.

3.4. Overexpression of DAAM1 predicts poor prognosis in BrCa patients

We next determined the prognostic values of *DAAM1* mRNA expression based on KM plotter. Among all BrCa patients, relapse-free survival (RFS), overall survival (OS), distance-metastasis-free survival (DMFS) and post-progression survival (PPS) data were available for 3951, 1402, 1746 and 414 patients, respectively. The survival curves revealed that patients with high expression of *DAAM1* mRNA exhibited shorter RFS ($P < 0.001$, Fig. 4A), OS ($P = 0.003$, Fig. 4B), DMFS ($P < 0.001$, Fig. 4C) and PPS ($P < 0.001$, Fig. 4D). To sum up, these online data implied that DAAM1 was a potential prognostic biomarker, and high *DAAM1* mRNA expression predicted worse prognosis of BrCa.

4. Discussion

Although therapeutic methods for BrCa have achieved great progress in recent years, the metastasis of malignance is always out of control and leads to over 90% BrCa-related deaths of all patients. Tumor metastasis is a complex, multi-step, and progressive pathological process, including epithelial-to-mesenchymal (EMT) transition (EMT), matrix adhesion, and pseudopod formation [22]. Undoubtedly, realization of these processes requires the rearrangement of cytoskeleton in tumor cells. Cytoskeleton, referring to the structure of protein fiber network in eukaryotic cells, contains microfilaments, microtubules and intermediate fibers. The microfilament is arranged by the spiral assembly of actin, which functions as an key controller in the formation of dynamically changing subcellular structures [23].

DAAM1, a crucial regulator in mediating the formation of microfilaments, is important to actin-dependent cellular behaviors. DAAM1 facilitates embryonic development, including the cardiac and neuronal systems [24–27] and regulates actin-induced mouse oocyte meiosis [6]. However, studies demonstrating the expression data of DAAM1 in clinical samples is unavailable yet. In this study, we first measured the difference of DAAM1 expression between BrCa and adjacent tissues. A notable increase in DAAM1 expression was found in BrCa tissues. We also observed upregulated expression of DAAM1 was notably correlated with lymph-node metastasis of BrCa which is consistent with the phenomenon showing the upregulated DAAM1 transcriptional level in metastatic BrCa tissues according to our recent research [21].

Our previous studies have reported that Wnt5a promotes tumor cells migration by regulating the Dvl/DAAM1/RhoA signaling pathway in BrCa and glioma cells [10,13,20]. DAAM1 serves as an effector of extracellular matrix (ECM) protein and is essential for cell haptotactic migration. Thus, no significant effect on tumor cells' migration was exhibited *in vitro* when without extracellular matrix inducer [13,14]. In this research, we further evaluated the effect of DAAM1 on BrCa cells migration capacity in xenograft model and the results validated that overexpressed DAAM1 largely stimulates BrCa cells lung metastatic extent *in vivo*. Furthermore, the prognostic value was next evaluated in KM plotter database online. The results revealed that overexpressed expression of *DAAM1* mRNA was correlated with unfavorable prognosis, including RFS, OS, DMFS, and PPS. Combined with our previous findings [13,14,20,21], we identify DAAM1 as a potential oncogene in BrCa and indicate that DAAM1 might be a promising therapeutic target in limiting the malignant progress of BrCa.

In summary, our results uncover the define role of DAAM1 in clinical samples and xenograft model as well as exact prognostic roles of *DAAM1* mRNA expression in BrCa patients. DAAM1 might be therapeutic target to manage the progress of BrCa and identified a biomarker for poor prognosis in BrCa patients.

Authors' contributions

YZ and JM conceived the study and participated in the study design, performance, coordination and manuscript writing. JM, BX, LH, ZX, YL, and TY carried out the assays and analysis. YZ, JM and BX revised the manuscript. All authors reviewed and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152736.

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