

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/173258/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Zhu, Kaitao, Li, Shiwei, Yao, Hongru, Hei, Jilong, Jiang, WenGuo , Martin, Tracey and Zhang, Shanyi 2024. Junctional adhesion molecular 3 (JAM3) is a novel tumor suppressor and improves the prognosis in breast cancer brain metastases via the TGF- β /Smad signal pathway. *Journal of Neuro-Oncology* 170 , pp. 331-345. 10.1007/s11060-024-04797-x

Publishers page: <http://dx.doi.org/10.1007/s11060-024-04797-x>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



Title page

Junctional adhesion molecular 3 (JAM3) is a novel tumor suppressor and improves the prognosis in breast cancer brain metastasis via the TGF- β /Smad signal pathway

Kaitao Zhu^{1,2}, Shiwei Li³, Hongru Yao^{1,2}, Jilong Hei^{1,2},
WenG Jiang,⁴ Tracey Martin⁴, Shanyi Zhang^{1,2,4}

1. Department of Neurosurgery, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China.
2. Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Guangdong-Hong Kong Joint Laboratory for RNA Medicine, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China.
3. Department of Neurosurgery, HeYou International Health System, Foshan, China.
4. Cardiff China Medical Research Collaborative (CCMRC), (Cardiff University - Peking University Cancer Institute and Cardiff University - Capital Medical University Joint Centre Biomedical Research), Cardiff University, School of Medicine, Cardiff, United Kingdom.

  Tracey Martin

MartinTA1@cardiff.ac.uk

  Shanyi Zhang

zsyscience@163.com

Abstract

Purpose The incidence of breast cancer brain metastasis (BCBM) is a deadly clinical problem, and exact mechanisms remain elusive. Junction adhesion molecule (JAM), a tight junction protein, is a key negative regulator of cancer cell invasion and metastasis.

Methods Junction adhesion molecular 3 (JAM3) expression in breast cancer was analyzed by bioinformatics method and confirmed by PCR, western blot, and immunofluorescence (IF) in cell lines. The effect of exogenous expression of JAM3 through lentivirus vectors on invasion, adhesion, and apoptosis was verified using transwell assay and flow cytometer. Differentially expressed genes (DEGs) were detected by RNA sequence and verified by q-PCR and Western blot. The effect of silencing JAM3 using siRNA was assessed by adhesion assay. Kaplan-Meier analysis was applied to calculate the impact of JAM3 expression and classic clinicopathologic characteristics on survival.

Results Bioinformatics analysis revealed that JAM3 expression was reduced in BCBM. Exogenous expression of JAM3 minimizes the ability to invade, adhesion and promotes apoptosis of breast cancer cells. Silencing JAM3 results in morphology-changing and recovering invasion and adhesion to ECMs and the TGF- β /Smad signal pathway may be involved. JAM3 predicts less metastasis and good survival in patients with BCBM. Statistical analysis examined the correlation between JAM3 expression in BCBM samples detected by IHC and the clinicopathological characteristics. Kaplan-Meier analysis indicated that a high expression level of JAM3 was associated with longer survival time.

Conclusion JAM3 can serve as a key negative regulator of breast cancer cell invasion, apoptosis, and brain metastasis, which may be linked to the TGF/Smad signal pathway. JAM3 has been anticipated to be a promising biomarker in the diagnosis and prognosis of breast cancer.

Introduction

Compared to primary brain tumors, brain metastases (BMs) occur four to ten times more often, making them the most prevalent type of intracranial tumor[1]. BMS are mainly derived from lung cancer, breast cancer, melanoma, and so on. In recent years, the development of tumor therapy has greatly improved the quality of life and extended the survival period. But it also leads to recurrence and metastasis, especially brain metastasis. The most prevalent malignancy in women is mammary tumors, and 20% of breast cancer patients will eventually develop brain metastases, making it one of the most difficult diseases to treat. In addition to some HER2⁺ patients who can benefit from trastuzumab (Herceptin) treatment, most of the patients have a one-year survival rate of < 20%[2]. At present, the main treatments for BMs of mammary tumors include surgery, radiotherapy, chemotherapy, etc., which can only improve symptoms but cannot effectively control tumor development. Therefore, brain metastases have become one of the biggest challenges facing clinicians, and it is critical to understand the basic mechanisms of BMs and develop new therapies.

One of the vital steps in BMs is to break the blood-brain barrier (BBB). It comprises tight junctions (TJS) between the brain capillary endothelium and its cells, the basal membrane, and the glial membrane, all surrounded by the astrocyte footplate[3]. The capillary endothelium and the tight junctions between the endothelium are the main structures of the BBB. There are two ways to enter the brain through the BBB: the paracellular pathway and the transcellular pathway, with the former accounting for more than 90%[4]. The proteins that function in the paracellular pathway include junction adhesion molecules (JAMs, including JAM-A, -B, -C, etc.), occludin, and claudin, all of which belong to tight adhesion proteins and form natural barrier[5]. Together, they maintain the BBB integrity and the stability of the brain environment. Peripheral tumor cells often need to inhibit or degrade the tight junction protein in the BBB to break through the barrier and enter the brain tissue, which has been reported by our research group.⁴ At present, research on TJs in brain metastases mainly focuses on proteins such as occludin and claudins, while there are few reports on junction adhesion molecules (JAMs) [6].

The pleiotropic roles of JAM family members in modulating adhesion, invasion, migration, angiogenesis, and metastasis of neoplastic cells indicated that JAMs may be an intriguing target for the future eradication of breast cancer, rather than simply serving as a moderate hallmark for classification[7]. One team initially reported that JAM-A could attenuate cell invasiveness in mammary tumor cell lines, which contributed to the increase in the assembly of focal adhesion moleandell as TJs[8]. These data suggest that JAM-A may function as a significant negative mediator of invasion and metastases of breast cancer. As for another member of the JAM family, JAM-B's expression spectrum in mammary tumors has been identified. The expression level of JAM-B as well as other TJ molecules in HER2⁺ breast tumors, including CLDN 5, CTNNAL1, ZO-1, and the PARD3 gene, was found to be unregulated compared to normal breast tissue[9]. Few studies on JAM3 in human breast tumors are available.

Previous studies have shown that JAM-C (also called JAM3) is involved in the migration of leukocytes and the constitution and maintenance of intercellular connections, suggesting that it may lead to the remodeling of endothelial connections[10]. Some researchers have demonstrated that

blocking antibodies against JAM3 can interfere with tumor growth in vivo and disrupt angiogenesis in vivo and in vitro[11]. We know that JAM3 on human leukocytes can interact with JAM-B in blood vessels to mediate leukocyte adhesion and transport. JAM3's impact on the oncogenes of breast cancer was clarified in a mouse model. Maternal high-fat (HF) diet intake during pregnancy resulted in an intergenerational increased mammary tumor risk in mouse offspring[12]. The upregulation of expression of JAM3 in offspring mice was detected, which suggests that JAM3 is potentially implicated in the tumorigenicity of breast cancer progression and has the potential to become a therapeutic target.

JAM3 has previously been identified as a biomarker for cervical cancer and is upregulated and associated with a higher incidence of cancer in the offspring of mice that ate a high-fat diet during pregnancy[13]. The expression and character of breast tumors remain unclear. Recently, Pieter found that JAM3 was down-regulated in triple-negative mammary tumors [14]. Such an observation prompted us to test whether JAM3 contributes to breast tumor brain metastasis. So, we hypothesize that JAM3 in mammary tumor cells may result in brain metastasis through its interaction with endothelial cells of the BBB and as a circulating biomarker to screen, diagnose, and even treat cancer.

For the first time, our study demonstrates a correlation between the expression level of JAM3, patient survival, and invasion as well as metastasis of breast cancer, suggesting that the tight junction protein JAM3 may be critically involved in the brain metastases of breast cancer. Our studies provide new insight into the molecular mechanisms responsible for the basic principle of mammary tumor cells breaking through the BBB and developing BMs. Also, we provide a new experimental basis and theoretical support for BMS diagnosis and therapy in breast cancer, and the findings may help develop more therapeutic strategies to eradicate tumors.

Materials and methods:

Patients and ethics

All mammary tumor and breast cancer brain metastasis samples were obtained from patients at Sun Yat-sen Memorial Hospital from March 2008 to December 2023, after surgery, and immediately placed in liquid nitrogen and kept in the freezer at -80 °C until needed. The Sun Yat-sen Memorial Hospital Research Ethics Committee approved all protocols and procedures, and the patient's informed consent was obtained.

Cell culture, chemicals, and lentiviral plasmids

The human mammary tumor cell lines BT-549, MDA-MB-468, MCF-7, MDA-MB-231(MDA231), BT-20, MDA-MB-468, Sk-Br-3, and MDA-MB-361, the human breast cancer brain metastasis cell line MDA-MB-231-brain-metastasis (MDA-231-BM) and the human breast cancer cell line MCF-10A were routinely cultured in Dulbecco's Modified Eagle's medium (DMEM; Sigma Aldrich Ltd.) mixed with 10% fetal bovine serum (FBS), penicillin and streptomycin (Sigma-Aldrich Ltd.). The ATCC-formulated RPMI-1640 Medium (Catalog No. 30-2001) was used to culture the human umbilical vein endothelial cell (HUVEC) line. All cell lines were procured from the American Type Collection (ATCC). The JAM3 overexpressed lentiviral plasmid vector clone containing hJAM3 ([NM_032801.4]) (pLV[Exp]-mCherry/Neo-CMV>3X FLAG/hJAM3) was made by Vector Builder (Vector Builder Inc.,

Guangzhou). The 293T cell lines were used to generate lentiviral particles. Subsequently, these lentiviral particles were used to seed and infect the mammary tumor and BCBM cell lines on 6-well plates. Following 72 hours, hygromycin (450 ug/ml) was used to select the cell lines. The vector containing siRNA targeting JAM3 and scrambled RNA was constructed by GenePharma (GenePharma Inc., Shanghai). Human breast cancer MCF-7 and T47D cells (5×10^3) were seeded in 24-well plates and vector-transduced in the presence of lipofectamine 3000 transfection reagent (L3000001, Thermo Fisher).

Transfection and transduction of tumor cells. In a six-well culture plate, 2×10^5 cells/well were seeded and Lipofectamine 2000 (Invitrogen) was used to transfect cells with three specific double strands of siRNA, as directed by the manufacturer. The transduction process for tumor cells involved transfecting cells in 24-well plates (0.5×10^5 cells/ml) and then transducing them with 5 μ g ML-1 polyethylene along with lentiviral particles.

Normal PCR and qPCR assays. Normal PCR and qPCR were finished as previously described[15]. The sequence of primers is shown in Supplementary Table 1. A Light Cyclor 480 from Roche was used for collecting and analyzing data.

Western Blot The process of protein isolation from cancer cells followed the steps outlined previously [15]. After quantification, proteins were transferred onto a PVDF (polyvinylidene fluoride) membrane (Millipore, USA). The membrane was detected with anti-human JAM3 rabbit polyclonal antibody (1:1000; ab224327, Abcam), BMP5 (1:1000; ab87627, Abcam), INHBA (1:1000; ab128958, Abcam), AMH (1:1000; ab313767, Abcam), ID2 (1:1000; ab90055, Abcam), INHBB (1:1000; ab128944, Abcam), GAPDH(1:2000; AG8015, Beyotime), and β -actin (1:2500; AF5003, Beyotime).

Apoptosis assay. The cells were treated as previously described[16] and analyzed immediately by flow cytometry (Accuri C6, BD, or Attune NxT, Thermo).

Migration and invasion assay. Previously published methods were followed to examine the ability of breast cancer cells to invade and migrate[17]. For quantification, we averaged the cell counts over all five fields of view in the microscope.

Immunofluorescence (IF) and immunohistochemical (IHC) analysis. Cells for immunofluorescence were tested as previously described[18]. After blocking in PBS containing 2% BSA for one hour at room temperature (RT), the samples were subjected to overnight incubation at 4 °C with antibodies specific for JAM3 (1:1000; ab224327, Abcam), TGF- β 1(1:1000; ab215715, Abcam). Alexa Fluor or HIP-conjugated secondary antibodies were incubated for 1 h at RT. After counterstaining the nuclei with DAPI, laser scanning confocal microscopy (LSM780, Zeiss)was employed to capture the images. DAB was used for immunohistochemistry as previously described. Under the $\times 40$ objective, the staining

fraction was determined according to JAM3-positive cell percentage and stain intensity in ten randomly selected fields. In the section, the percentage of tumor cells that stained positively was as indicated below, no positive cells; 1, < 25%; 2, 25-50%; 3, 50-75%; 4 >75%. The cells that were stained at different intensities were noted as 0(unstained), 1(light brown), 2(brown), and 3(dark brown). The formula for calculating the staining index (SI): SI= staining intensity X proportion of positively stained cells. TGF- β 1 was evaluated by SI-stained method with scores ranging from 0 to 12, cut-off point ≤ 3 And cut-off point >3 . As previously reported, the staining score for TGF- β 1 was calculated by considering the extent of tumor area covered and the proportion of BMP5 positive staining.

Adhesion to extracellular matrix (ECM) proteins and vascular endothelial cells As previously described[19], the 24-well plates were coated with fibronectin (10 ug/ml), type I collagen (20 ug/ml), and vitronectin (0.5 ug/ml), and were blocked for an hour with 20% BSA at 37°C following rising, with 3% BSA as a negative control. Each well was supplemented with 50,000 cells, incubated at 37°C for half an hour, rinsed, fixed adherent cells, stained with crystal violet, and scored per 10 X field. HUVECs were cultured on 24-well plates until confluent. Each well was supplemented with 50,000 tumor cells. Two hours later, scores were recorded for each 10x magnification of the field of view (Leica MZ10F stereo microscope)after rinsing the cells and fixing the adhering cells with 10% formalin.

In vitro transendothelial migration and BBB transmigration

A method that was previously outlined was implemented to evaluate transendothelial migration[20]. Throughout the night, fibronectin was applied to transwell inserts that had pores measuring 8 mm. Following the seeding of 50,000 HUVECs in the top chamber of the insert, they were cultured until they reached confluence. After 24 h, 5×10^5 tumor cells were added to each well, followed by fixing them in 10% formalin and scoring them with a Leica MZ10F stereomicroscope.

In addition, primary human astrocytes were co-cultured with primary HUVECs on each side of a fibronectin-coated insert with pore sizes of 8 mm, for BBB assay. After incubating 20,000 primary human astrocytes on the membrane surface for a half hour at 37°C, HUVECs were introduced and grown to confluence following the protocol of the transendothelial migration experiment. Twenty-four hours following confluence, 5×10^5 cancer cells were seeded into the top chamber of the inserts. At 10-field intervals for 48 hours, the total count of cherry-fluorescent cancer cells that crossed the endothelial and astrocyte layers was determined. A 10% formalin solution was used to fix the migratory cells that emit cherry-fluorescent stain (Leica MZ10F stereo microscope).

RNA-seq and gene set enrichment analysis

JAM3 overexpressing cells and control MDA-231 cells (n = 3 per group) were prepared for RNA sequencing. The 2100 Agilent 2100 Bioanalyzer or RNA-specific agarose electrophoresis was utilized to verify the integrity, while nanodrop (Thermo Scientific, Waltham, Massachusetts, USA) was utilized to assess the concentration and purity. Afterward, total RNA (>1ug)was selected and the library quality was examined with the NEBNext Ultra II RNA Library Prep Kit for Illumina Kit (New England Biolabs Inc;

Ipswich, Massachusetts, USA) Agilent 2100 Bioanalyzer (Agilent Technologies Inc, California, USA) and Agilent High Sensitivity DNA Kit (Agilent Technologies Inc, California, USA, 5067-4626). After homogenization, the multiplexed DNA libraries were combined in equal volumes. An Illumina sequencer was used to sequence the mixed libraries in PE150 mode after they had been progressively diluted and quantified. Subsequently, Suzhou PANOMIX Biomedical, Tech Co., Ltd. (Suzhou, China) conducted the transcriptome sequencing.

Differentially expressed genes (DEGs, $P < 0.05$, fold change > 1.5) were identified using the DESeq software (V 1.20.0). We used topGO for gene ontology (GO) analysis; cluster Profiler (v3.4.4) for KEGG pathway enrichment analysis, and GSEA software for gene set enrichment analysis (GSEA).

Statistics and reproducibility. SPSS v.20.0 (SPSS) was utilized for statistical analysis. For in vitro experiments, the P-values were computed utilizing an independent sample t-test. To assess the interrelationships of the variables, we conducted a Spearman ordinal correlation analysis. In all statistical analyses, two-tailed P values were used. Three independent repetitions of the experiments produced comparable findings, as shown by immunoblotting, IF, and IHC tests, alongside the representative data in the figures. Overall survival of BCBM, survival rates, and associations with JAM3 expression were assessed using Kaplan-Meier (KM) methods and log-rank tests.

Results

JAM3 expression was reduced in invasive and metastatic mammary tumors, and decreased expression of the protein JAM3 indicates a poor prognosis in breast cancer brain metastasis

The JAM3 expression characteristics in breast cancer were further elucidated through differential analysis of normal and cancerous breast tissue in the TCGA and GEO databases. When comparing cancerous to normal breast tissue, these data demonstrated a decrease in JAM3 expression in the former (Figures 1a, 1b, and 1c). We paired TCGA and CGGA clinical data with expression data to find out how JAM3 affects mammary tumor prognosis. The KM analysis showed that breast cancer patients with low JAM3 expression exhibited an unfavorable prognosis (Fig. 1d, S1a).

The JAM3 expression levels in breast cancer cell lines were detected by measuring JAM3 mRNA utilizing both conventional PCR and real-time PCR in 10 mammary tumor cell lines isolated from humans that have unique potential for metastasis: MDA-231-BM, MDA231, BT549, BT20, MDA468, MDA361, SKBR3, ZR75.1, T47D, and MCF-7. Fig.1e illustrates a representative ethidium bromide-stained agarose gel of JAM3 mRNA expression. As expected, in human breast cell MCF-10, the level of JAM3 is up-regulated compared to the other human breast cancer cell lines. Among the types of luminal breast cancer, MCF-7, T47D, ZR75.1, SKBR3, and MDA361 mostly expressed a high level of JAM3. The type of basal an, including MDA468 and BT20, also expresses JAM3. As for the type of basal B with metastatic potential: BT549, MDA231, and mammary tumor brain metastatic cell line MDA-231-BM, JAM3 seldom could be detected. These data suggest that JAM3 levels may contribute to the distinct invasiveness of human breast cancer cells. Next, we examined whether JAM3 localization at tight junctions is affected by its variable expression in these cell lines. By immunofluorescence, we found

that in MFC-7 and T47D cells, JAM3 was localized to tight junctions as much as ZO-1, whereas, in MDA231, we did not observe the usual junctional expression of JAM3 (Figure 1f). Quantitative analysis of these data showed that JAM3 expression reached the highest levels in T47D cells with MCF-7 > BT549 > MDA231 (Figure 1f). Together, these data suggest that JAM3 expression is negatively correlated with the well-established tendency of these cell lines to metastasize

Exogenous expression of JAM3 in Mammary tumor cells reduces their potential to migrate, invasion, adhesion, and transmigration across ECMs and vascular endothelial cells and promotes apoptosis of breast cancer cells.

To test whether JAM3 expression could affect breast cancer cells MDA231, BT549, and MDA-231-BM, which all express low levels of JAM3; we ectopically overexpressed JAM3 in these cells through lentivirus plasmid transfection. A normal PCR (Fig. 2a) and western blot (Fig. 2b, 2c) analysis indicated a substantial upregulation of JAM3 mRNA and protein in those three cell lines after transfection. Immunofluorescence analysis showed JAM3 expression at the tight junction was increased, while exogenous expression of JAM3 was also increased (Fig. 2d). Interestingly, we found that another tight junction molecular ZO-1 expression was upregulated after exogenous expression of JAM3 (Fig. 2d). These data offer evidence that overexpression of JAM3 in mammary tumor cells may influence the level of ZO-1 protein and enforce their junctional properties.

Given that human breast tissue contains abundant collagen matrix, we conducted a tactile migration assay using collagen as the matrix. The transwell tactile migration experiment on collagen provided additional evidence of JAM3's impact on breast cancer cells. As shown in (Figure 2d), parental MDA-231-BM cells migrated to a high degree, while cells overexpressing JAM3 migrated to a lesser extent (Fig. 2d). These results demonstrate that JAM3 inhibits the motility of breast cancer cells that are extremely migratory.

Enhanced invasion is one of the hallmarks of tumor cell metastasis. Subsequently, we investigated the possibility that JAM3 upregulation would inhibit the invasiveness of tumor cells. The transwell collagen gel invasion experiment was conducted with a conditioned medium serving as an attractant. We identified that MDA231 cells could effectively invade collagen, while overexpression of JAM3 inhibited this effect. Quantitative analysis data showed that JAM3 inhibited the invasion of tumor cells through collagen gel. Afterward, we tested whether the ability of JAM3 is specific to some types of cancer cells. To achieve this, we confirmed it using MDA231 and BT549 cells in an invasion assay. We found that overexpression of JAM3 also blocked these two cell invasions (Fig. S2). These results indicate that exogenous JAM3 inhibits the invasive capacity of mammary tumor cells.

Cancer cell adhesion to and interaction with the vasculature of specific organs are essential steps in the metastasis cascade. JAM3 may be important in metastatic cancer cells as a molecule involved in cell-cell adhesion. To test it, we performed an adhesion assay to investigate the interaction between JAM3 and the vascular matrix proteins fibronectin (FN), collagen (CL), and vitronectin (VN), with bovine serum albumin (BSA) as negative control. We identified that MDA-231-BM cell's overexpression JAM3 could more easily adhere to CL and VN than parental cells (Fig. 2f). Whereas, it could not make a difference when they both adhered to FN (Fig. 2f). Similarly, MDA231 cells showed an identical result.

This suggests that JAM3 influences the crosstalk between cancer cells and the vascular matrix and is matrix-specific (Fig. 2f).

Next, we investigated the mutual effect between cancer cells and vascular endothelial cells and how they adhere to each other. Human breast cancer cells (MDA-231-BM) were seeded on the human umbilical vein endothelial cell (HUVEC). We found that fewer MDA-231-BM cells overexpressing JAM3 could adhere to the HUVEC cells, which hints that JAM3 makes cancer cells less easily stick to these cerebral microvascular endothelial cells (Fig. 2g).

The migration of tumor cells across the BBB is a key event in the metastasis of cancer cells to the brain. Therefore, a major factor in breast cancer brain metastasis (BCBM) may be the ability of cancer cells to cross the BBB. We built one model of BBB in vitro using a two-cell system including human astrocytes and human cerebral microvascular endothelial cells (Fig. 2h). We first tested the integrity of the BBB through a permeability assay with FITC dextran and valued the resistance of cell layers in an 8.0-mm insert. When the resistance comes to $350 \Omega\text{cm}^2$, it means that the model of BBB is feasible. We found that mammary tumor cells could invade the two-cell model more easily than those cells expressing JAM3 (Fig. 2h). We could get the same results in MDA-231-BM and BT-549 cells (Fig. 2h). These results suggest that JAM3 makes breast cancer cells less easily break through astrocyte-D3 cell layers, so we can conclude that JAM3 may hinder cancer cells from passing through the BBB in vitro.

Detection of early and late apoptosis by flow cytometry (FCM) assay through annexin V and PI staining was used to evaluate whether JAM3 could induce mammary tumor cell death via apoptosis. FCM analysis indicated that the apoptosis rates of MDA231^{JAM3} and control cells were, respectively, 4.9% and 1.5%. The result indicates that JAM3 could induce apoptosis in breast cancer cells, which may contribute to JAM3 restricting metastasis.

Silencing JAM3 in MCF-7 cells results in morphology-changing and recovering migration, invasion, and adhesion to ECMs

According to our findings, JAM3 is highly expressed in MFC-7 and T47D cells, although their capacity for invasion is weak (data not showing). Additionally, the effects of silencing-JAM3 on the morphology of cancer cells, invasion and adhesion to the ECM, and transmigration across the vascular endothelial cells were investigated. JAM3-specific siRNAs specifically reduce JAM3 levels in MCF-7 cells. (Fig. 3a), and western blot result also demonstrated a significant decrease in JAM3 protein levels (Fig. 3b). Interestingly, we found that silencing JAM3 may increase the mRNA and protein levels of integrin $\beta 3$, which belongs to an important cell adhesion molecular family and plays a key function in the progression and invasion of cancer (Fig. 3b, 3c). Additionally, after JAM3-silencing breast cancer cells achieved the confluence at low density, it was observed that these cells were round and tended to aggregate (Figure 3D). Comparatively, the parental cells had an iconic fibroblast-like morphology (Figure 3D). Our observation hints that JAM3 affects the morphology of tumor cells and reduces their aggressiveness.

We examined whether JAM3 knockdown affected cancer cells' capacity for invasion. This effect

was further confirmed by hypotactic and random transwell invasive assays on collagen. As shown in Fig. 3e, parental MCF-7 and T47D cells migrated to a low extent, whereas cells with downregulated JAM3 expression migrated much more. Quantitation of these data demonstrated a significantly attenuated invasive capacity of MCF-7 and T47D cells ($p < 0.001$) compared to that of cells with downregulated JAM3 levels (Fig. 3e).

We performed an adhesion assay to study the effect of silencing JAM3 on mammary tumor cells adhering to the vascular matrix protein. We found that after JAM3 silencing, MCF-7 cells adhered more easily to CL and VN than parental CMF-7 cells (Fig. 3g). Similarly, T47D cells showed us an identical result. This suggested that silencing JAM3 benefits the crosstalk between cancer cells and the vascular matrix (Fig. 3g). Interestingly, this effect could be neutralized by an ITG β 3-blocking antibody (Fig. 3g).

Next, we examined the mutual effect of silencing JAM3 on the ability of vascular endothelial cells. We found that after silencing JAM3, more MCF-7 cells adhered to the HUVEC cells, which hints that JAM3 makes cancer cells less easily stick to these cerebral microvascular endothelial cells (Fig. 3f). These results suggested that silencing JAM3 could help cancer cells invade or inhibit the motility of migratory breast cancer cells and invasion.

JAM3 modulates breast cancer cell invasion and adhesion to the ECM and HUVEC via the TGF- β /Smad signal pathway

We conducted transcriptomic RNA-sequencing analysis of JAM3-overexpressed MDA231 cells and parent cells, identified 494 differentially expressed genes (DEGs), and further analyzed the related pathways by GO, KEGG, and GSEA, to further reveal the signaling pathways involved in JAM3 (Fig. 4a, 4b, 4c, 4d). QPCR verified the transcriptomic results suggesting that JAM3 inhibited BMP5, INHBA, and AMH, ID2 and activated both FST and INHBB in comparison to the control group. We found that the activity of the TGF- β signaling pathway was significantly reduced in mammary tumor cells with overexpressed JAM3 (Fig. 4f). At the transcription level, the same results were obtained from WB results (Fig. 4g). These results suggest that JAM3 may negatively regulate the TGF- β /Smad signaling pathway to influence the invasion and apoptosis of breast cancer cells.

JAM3 predicts less metastasis in patients with breast cancer and good survival in patients with breast cancer brain metastases

To further examine the level of JAM3 and TGF- β 1 protein expression, we examined them in normal breast tissue, primary breast carcinomas, invasive mammary tumors, and brain metastases using immunohistochemical (IHC) staining. As shown in Fig. 5a, high levels of JAM3 were present in 16 of 58 primary breast tumors and 9 of 39 breast cancer brain metastasis. In contrast, JAM3 was marginally detectable in most invasive breast tumors and undetectable in most brain metastatic tumors. Interestingly, JAM3 is highly expressed in normal brain tissues. Furthermore, more intense TNF- β 1 staining was seen in primary and brain metastatic breast tumors than in normal breast tissues (Fig. 5a).

Taken together, these observations suggest that low levels of JAM3 expression and high levels of

TNF- β 1 is associated with the clinical development of primary and metastatic breast tumors.

Statistical analysis was performed to examine the correlation between JAM3 detected by IHC and the clinicopathological characteristics of breast tumors. As shown in Table 5b, the expression level of JAM3 protein in breast cancer patients was not correlated with the patient's age and the expression levels of estrogen receptor, progesterone receptor, and Her2. In contrast, the expression of JAM3 in mammary tumor brain metastasis was closely related to the brain as the first metastatic site ($P = 0.0008$). Overall, the expression of the JAM3 protein was correlated with brain metastasis.

Survival analysis. The expression of JAM3 protein in breast cancer was significantly correlated with the patient's survival time ($P < 0.01$), with a correlation coefficient of 0.326, clearly indicating that a high expression level of JAM3 was associated with longer survival time.

Discussion

The underlying pathogenic mechanism of breast cancer brain metastasis (BCBM) continues to be vaguely comprehended[21]. The entire metastatic cascade is first triggered by the uncontrolled growth of tumor cells, detachment from their neighbors, and invasion into the tissues surrounding them, especially the microvasculature. The aggressive phenotype results from the enhanced migratory and invasive abilities of cancer cells. Tumor cells with more mobility permeate through various barriers, including tight junctions (TJs), and are attributed to micrometastases and distant metastases[22]. Here, we show that JAM3, one of the TJs, is deemed to be implicated in mammary tumor migration, invasion, and transmigration of vascular endothelial cells. As suggested by our results, exogenous overexpression of JAM3 substantially impaired the migratory and invasive characteristics in highly metastatic breast cancer cells, whereas silencing JAM3 in less malignant breast cancer cells enhanced those abilities to invade. Additionally, up-regulation of JAM3 reduced adhesion to microvasculature and extracellular matrices (ECM), which may correlate with the loss of integrin β 3 expression regulated by JAM3. Additionally, we found that JAM3 may restrain the TGF/Smad signal pathway and trigger apoptosis in mammary tumor cells. Collectively, we have shown that reduced expression of JAM3 seems to be responsible for the invasive, adhesive, and vascular endothelial transmigration behavior of metastatic breast cancer cells. This is the first evidence that we are aware of linking the inverse association of JAM3 in human breast cancer cells to invasion and metastatic capacities.

It is conceivable that down-regulation or loss of JAMs is implicated in the disruption of tight junctions in tumor cells and attributed to the consequences of metastases[23, 24]. Studies identified JAM3 as necessary for both adhesions to endothelial cells and extravasation into the vasculature[25]. Several follow-up studies across a wide array of cancers have likewise implicated JAM3 in metastasis[23]. Evidence illustrates that JAM3 could have disparate roles in different types of tumors. Similarly, JAM3, as a novel tumor suppressor with epigenetic reduction, was shown to be effective in reducing colorectal cancer (CRC) and could be used as a potential non-invasive biomarker for CRC diagnosis[26]. Furthermore, JAM3 expression has been revealed to be down-regulated in gastric adenocarcinoma, and in tumor cells lacking JAM3, increasing JAM3 expression enhances the tight junctional barrier[24]. However, the potential role of JAM3 remains under-discussed in breast cancer literature, particularly

those dealing with metastatic breast cancer. We demonstrated the decrease in JAM3 expression in the metastatic breast cancer cell lines MDA-231, MDA-231-BM, and BT549 using normal PCR, real-time PCR, and western blotting, whereas a significantly high level of expression was noted in the less aggressive MCF-7 and T47D cells. Our results thus confirm and extend the findings of Pieter, who reported the downregulation of JAM3 in triple-negative breast cancer (TNBC)[14].

JAMs have a key function in the maintenance of tight junction(TJ) integrity. Research has demonstrated that TJ disruption triggers a cascade of molecular events that contribute to cancer cell invasion, migration, and metastasis[23]. Meghan U. found that TJs are disrupted when JAM-A is silenced in metastatic cells. On the contrary, JAM-A upregulation contributes to the formation of TJs[8]. Consistent with the above findings, we showed that exogenous upregulation of JAM3 in metastatic breast cancer cell lines contributed to the decrease in migration, invasiveness, and mobility of cancer cells. Conversely, silencing the expression of JAM3 in less malignant cells (MCF-7 and T47D) resulted in the enhancement of invasion and mobility. In the meantime, JAM3 knockdown changed the shape of tumor cells from spindle-shaped to spherical. Our findings align with a previous investigation that found that silencing JAM-A with siRNA increased the migratory activity of malignant breast cancer cells[8]. Thus, JAM3 could be a key player in triggering vascular endothelial cell transmigration and invasion by breast cancer cells. Accordingly, invasive breast cancer may be predicted by the loss of JAM3, a protein that prevents tumor invasion and brain metastases.

The mechanism underlying tumor metastasis is not well understood. One of the related explanations is deemed to be associated with the adhesion of tumor cells to vascular endothelial cells, which requires the interaction of cell surface integrin with its legends in the ECM[27]. Integrins have been implicated in the attachment of tumor cells to vascular endothelial cells and are involved in tumor metastasis[28]. Herein, we found several integrins correlated to cancer metastasis in JAM3-overexpressing cancer cells. Among them, integrin $\beta 3$ (ITG $\beta 3$) was down-regulated. Meanwhile, the ITG $\beta 3$ was up-regulated in cells after JAM3 silencing. These results suggested that JAM3 may be related to ITG $\beta 3$. This concept is supported by the phenomenon that integrin $\beta 3$ is involved in the adherence of tumor cells to vascular endothelial cells and the BCBM[29]. Orthotic models of breast cancer show enhanced metastasis when $\alpha v\beta 3$ is activated[30]. Clinically, $\alpha v\beta 3$ is increased in cases of breast cancer that have metastasized to the bone [31]. According to our analysis, the impact of JAM3 on tumor cell adhesion to endothelial cells may be a reflection of the interaction between ITG $\beta 3$ and certain legends. Consequently, we used a blocking antibody specific to ITG $\beta 3$ to evaluate the attachment of tumor cells to vascular endothelial cells and the ECM. We found that the ITG $\beta 3$ antibody showed a significant negative effect on tumor adhesion and hindered breast cancer cells from adhering to vascular endothelial cells. As suggested by our study results, integrin $\beta 3$ is one of the downstream effectors of JAM3-silencing that influences the metastatic activity of breast cancer cells.

The detailed mechanism underlying JAM3 directly regulating ITG $\beta 3$ expression is unclear. Some studies indicate that the transcriptional factor MYC drives breast cancer metastasis to the brain and is deemed to be implicated in regulation as an intermediary agency[32]. Three CACGTG (MYC) consensus sites are thought to be located in the proximal promoter region of the human integrin $\beta 3$

gene, and under various conditions, MYC can suppress the transcription of ITG β 3[33]. Furthermore, Zhang demonstrated that JAM3 could activate MYC through the WNT/ β -catenin pathway in leukemia[34]. Therefore, we hypothesized that JAM3 restricted the adhesion and migratory capabilities of breast cancer cells by reducing the transcriptional activity of ITG β 3 via the transcriptional factor MYC. Additional thorough research is required to assess the involvement of JAM3 and MYC in integrin β 3-dependent regression of breast cancer. Although we have not yet determined whether JAM3 and ITGB3 have a direct effect, these studies suggest that JAM3 could be an effective therapeutic target for the treatment of brain metastases caused by breast cancer.

The transforming growth factor β (TGF- β) pathway is involved in regulating cell proliferation, differentiation, apoptosis, and other important functions[35], TGF- β 1 has a key function in breast cancer progression in patients with organ metastases, and targeted combination therapy resulted in a complete suppression effect[36]. Our results indicated that JAM3 is correlated with the TGF- β 1 signal pathway leading to tumor progression. We found that JAM3 may restrain the TGF/Smad signal pathway protein, including BMP5, INHBA, AMH, ID2, FST, and INHBB, and trigger apoptosis in breast cancer cells. Based on our results, we emphasize the importance of performing comprehensive molecular profiling to develop atypical treatments that could lead to better prognosis for patients with BCBM.

In addition, we also detected JAM3 expression in several breast cancer tissue specimens using immunohistochemistry analysis. Among 58 breast cancer tissues, 16 cases showed moderate or high staining of JAM3 detection. This supports the idea that JAM3 may perform a notable function in the ontogenesis of breast cancer brain metastasis. In addition, the relationship between JAM3 staining and clinical characteristics of patients was analyzed. We found that JAM3 expression was significantly related to the clinical stage and T, N, and M classification of breast cancer patients, but was not related to the age, estrogen receptor, and progesterone of breast cancer patients. Receptor status and ErbB-2 expression were irrelevant. This strongly suggests that JAM3 can be an independent indicator for identifying subgroups of breast cancer patients with a more aggressive disease. In addition, the cumulative 5-year survival rate of patients in the JAM3 low expression group was 5.1%, which was significantly lower than the 55.7% of patients in the JAM3 high expression group, indicating the possibility of using JAM3 as an indicator for prognosis and survival.

These studies provide new insights into the key role of JAM3 in the onset and advancement of human BCBM. Nevertheless, whether JAM3 expression parallels the process of brain metastasis or can serve as an indicator of cancer progression remains to be clarified. Additionally, our results suggest the need for more detailed analyses of different animal models to establish if JAM3 may affect BCBM as well.

In conclusion, our study highlights a previously unrecognized function for JAM3 in BCBM and points to JAM3 and TGF- β 1 as potential targets for this devastating disease. Importantly, our analysis proved that JAM3 is a promising biological marker for breast cancer, especially BCBM. A negative correlation exists between JAM3 and the clinical features and survival rate of invasive breast cancer. Additionally, JAM3 suppressed breast cancer cell migration, invasion, adhesion to, and transmigration in the ECM and vascular endothelial cells. Furthermore, the JAM3 expression has been linked to the TGF/Smad signal pathway,

which modulates breast cancer behavior and impacts the treatment response of breast cancer. Eventually, Our results point to a previously unrecognized role of JAM3 in BCBM and suggest that JAM3 may be a useful biomarker to identify poor-prognosis patients with breast cancer.

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

Acknowledgments We would like to thank all the participants of this study for their contribution to this research.

Author contributions SZ and MT designed the research. KZ, SW, and HY performed the experiments and analyzed the data. JL participated in the data collection and analyses. SZ drafted the manuscript, MT edited the manuscript.

Funding This study was supported by China Scholarship Council (No.201706385069), Cancer Research Wales(WGJ2013), the CardiffChina Medical Scholar-ship (CCMRC2017), the Key Research and Development Plan Project of Guangzhou (2023B03J0079), Natural Science Foundation of Guangdong Province (2018A0303130193, 2022A1515012393), the General Program of Medical Scientific Research Foundation of Guangdong Province (A2016072), and the Qihang Scientific General Project of Sun Yat-Sen Memorial Hospital (YXQH201803).

Competing interests The authors declare no competing interests.

Reference:

1. Zhao X, Powers S (2017) New Views into the Genetic Landscape of Metastatic Breast Cancer. *Cancer Cell* 32: 131-133 doi:10.1016/j.ccell.2017.07.011
2. Pan JK, Lin CH, Kuo YL, Ger LP, Cheng HC, Yao YC, Hsiao M, Lu PJ (2021) MiR-211 determines brain metastasis specificity through SOX11/NGN2 axis in triple-negative breast cancer. *Oncogene* 40: 1737-1751 doi:10.1038/s41388-021-01654-3
3. Li Y, Liu C, Chen Z, Lin H, Li X (2024) Netrin-1 protects blood-brain barrier (BBB) integrity after cerebral ischemia-reperfusion by activating the Kruppel-like factor 2 (KLF2)/occludin pathway. *J Biochem Mol Toxicol* 38: e23623 doi:10.1002/jbt.23623
4. Qi D, Lin H, Hu B, Wei Y (2023) A review on in vitro model of the blood-brain barrier (BBB) based on hCMEC/D3 cells. *J Control Release* 358: 78-97 doi:10.1016/j.jconrel.2023.04.020
5. Hosoya KI, Takashima T, Tetsuka K, Nagura T, Ohtsuki S, Takanaga H, Ueda M, Yanai N, Obinata M, Terasaki T (2000) mRNA expression and transport characterization of conditionally immortalized rat brain capillary endothelial cell lines; a new in vitro BBB model for drug targeting. *J Drug Target* 8: 357-370 doi:10.3109/10611860008997912
6. Jin J, Cui Y, Niu H, Lin Y, Wu X, Qi X, Bai K, Zhang Y, Wang Y, Bu H (2024) NSCLC extracellular vesicles containing miR-374a-5p promote leptomeningeal metastasis by influencing blood-brain barrier permeability. *Mol Cancer Res* doi:10.1158/1541-7786.MCR-24-0052
7. Martin TA (2014) The role of tight junctions in cancer metastasis. *Semin Cell Dev Biol* 36: 224-231 doi:10.1016/j.semcdb.2014.09.008
8. Naik MU, Naik TU, Suckow AT, Duncan MK, Naik UP (2008) Attenuation of junctional adhesion molecule-A is a contributing factor for breast cancer cell invasion. *Cancer Res* 68: 2194-2203 doi:10.1158/0008-5472.CAN-07-3057
9. Tokes AM, Szasz AM, Juhasz E, Schaff Z, Harsanyi L, Molnar IA, Baranyai Z, Besznyak I, Jr., Zarand A, Salamon F, Kulka J (2012) Expression of tight junction molecules in breast carcinomas analyzed by array PCR and immunohistochemistry. *Pathol Oncol Res* 18: 593-606 doi:10.1007/s12253-011-9481-9
10. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, Nash GB, Chavakis T, Albelda SM, Rainger GE, Meda P, Imhof BA, Nourshargh S (2011) The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat Immunol* 12: 761-769 doi:10.1038/ni.2062
11. Lamagna C, Hodivala-Dilke KM, Imhof BA, Aurrand-Lions M (2005) Antibody against junctional adhesion molecule-C inhibits angiogenesis and tumor growth. *Cancer Res* 65: 5703-5710 doi:10.1158/0008-5472.CAN-04-4012
12. Nguyen NM, de Oliveira Andrade F, Jin L, Zhang X, Macon M, Cruz MI, Benitez C, Wehrenberg B, Yin C, Wang X, Xuan J, de Assis S, Hilakivi-Clarke L (2017) Maternal intake of high n-6 polyunsaturated fatty acid diet during pregnancy causes a transgenerational increase in mammary cancer risk in mice. *Breast Cancer Res* 19: 77 doi:10.1186/s13058-017-0866-x
13. Eijssink JJ, Lendvai A, Derogowski V, Klip HG, Verpooten G, Dehaspe L, de Bock GH, Hollema H, van Criekinge W, Schuurin E, van der Zee AG, Wisman GB (2012) A four-gene methylation marker panel as a triage test in high-risk human papillomavirus positive patients. *Int J Cancer* 130: 1861-1869 doi:10.1002/ijc.26326
14. Segaert P, Lopes MB, Casimiro S, Vinga S, Rousseeuw PJ (2019) Robust identification of target genes and outliers in triple-negative breast cancer data. *Stat Methods Med Res* 28: 3042-3056 doi:10.1177/0962280218794722
15. Zhang SY, Li JL, Xu XK, Zheng MG, Wen CC, Li FC (2011) HMME-based PDT restores expression and function of transporter associated with antigen processing 1 (TAP1) and surface presentation of MHC class I antigen in human glioma. *J Neurooncol* 105: 199-210 doi:10.1007/s11060-011-0584-7
16. Doosti Z, Ebrahimi SO, Ghahfarokhi MS, Reisi S (2024) Synergistic effects of miR-143 with miR-99a inhibited cell proliferation and induced apoptosis in breast cancer. *Biotechnol Appl Biochem* doi:10.1002/bab.2592
17. Wang WW, Chen B, Lei CB, Liu GX, Wang YG, Yi C, Wang YY, Zhang SY (2017) miR-582-5p inhibits invasion and migration of salivary adenoid cystic carcinoma cells by targeting FOXC1. *Jpn J Clin Oncol* 47: 690-698 doi:10.1093/jjco/hyx073
18. Zhang S, Ma H, Zhang D, Xie S, Wang W, Li Q, Lin Z, Wang Y (2018) LncRNA KCNQ1OT1 regulates proliferation and cisplatin resistance in tongue cancer via miR-211-5p mediated Ezrin/Fak/Src signaling. *Cell Death Dis* 9: 742 doi:10.1038/s41419-018-0793-5
19. Wu Y, Liu H, Sun Z, Liu J, Li K, Fan R, Dai F, Tang H, Hou Q, Li J, Tang X (2024) The adhesion-GPCR ADGRF5 fuels breast cancer progression by suppressing the MMP8-mediated antitumorigenic effects. *Cell Death Dis* 15: 455 doi:10.1038/s41419-024-06855-8
20. Surve CR, Duran CL, Ye X, Chen X, Lin Y, Harney AS, Wang Y, Sharma VP, Stanley ER, Cox D,

- McAuliffe JC, Entenberg D, Oktay MH, Condeelis JS (2024) Signaling events at TMEM doorways provide potential targets for inhibiting breast cancer dissemination. *bioRxiv* doi:10.1101/2024.01.08.574676
21. Jin X, Demere Z, Nair K, Ali A, Ferraro GB, Natoli T, Deik A, Petronio L, Tang AA, Zhu C, Wang L, Rosenberg D, Mangena V, Roth J, Chung K, Jain RK, Clish CB, Vander Heiden MG, Golub TR (2020) A metastasis map of human cancer cell lines. *Nature* 588: 331-336 doi:10.1038/s41586-020-2969-2
 22. Wu D, Deng S, Li L, Liu T, Zhang T, Li J, Yu Y, Xu Y (2021) TGF-beta1-mediated exosomal Inc-MMP2-2 increases blood-brain barrier permeability via the miRNA-1207-5p/EPB41L5 axis to promote non-small cell lung cancer brain metastasis. *Cell Death Dis* 12: 721 doi:10.1038/s41419-021-04004-z
 23. Peng J, Chen Y, Yin A (2024) JAM3 promotes cervical cancer metastasis by activating the HIF-1alpha/VEGFA pathway. *Bmc Womens Health* 24: 293 doi:10.1186/s12905-024-03127-7
 24. Hajjari M, Behmanesh M, Sadeghizadeh M, Zeinoddini M (2013) Junctional adhesion molecules 2 and 3 may potentially be involved in the progression of gastric adenocarcinoma tumors. *Med Oncol* 30: 380 doi:10.1007/s12032-012-0380-z
 25. Mochida GH, Ganesh VS, Felie JM, Gleason D, Hill RS, Clapham KR, Rakiec D, Tan WH, Akawi N, Al-Saffar M, Partlow JN, Tinschert S, Barkovich AJ, Ali B, Al-Gazali L, Walsh CA (2010) A homozygous mutation in the tight-junction protein JAM3 causes hemorrhagic destruction of the brain, subependymal calcification, and congenital cataracts. *Am J Hum Genet* 87: 882-889 doi:10.1016/j.ajhg.2010.10.026
 26. Zhou D, Tang W, Zhang Y, An HX (2019) JAM3 functions as a novel tumor suppressor and is inactivated by DNA methylation in colorectal cancer. *Cancer Manag Res* 11: 2457-2470 doi:10.2147/CMAR.S189937
 27. Benedetti A, Turco C, Gallo E, Daralioi T, Sacconi A, Pulito C, Donzelli S, Tito C, Dragonetti M, Perracchio L, Blandino G, Fazi F, Fontemaggi G (2024) ID4-dependent secretion of VEGFA enhances the invasion capability of breast cancer cells and activates YAP/TAZ via integrin beta3-VEGFR2 interaction. *Cell Death Dis* 15: 113 doi:10.1038/s41419-024-06491-2
 28. Slack-Davis JK, Atkins KA, Harrer C, Hershey ED, Conaway M (2009) Vascular cell adhesion molecule-1 is a regulator of ovarian cancer peritoneal metastasis. *Cancer Res* 69: 1469-1476 doi:10.1158/0008-5472.CAN-08-2678
 29. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmed L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D (2015) Tumour exosome integrins determine organotropic metastasis. *Nature* 527: 329-335 doi:10.1038/nature15756
 30. Yoon S, Yang H, Ryu HM, Lee E, Jo Y, Seo S, Kim D, Lee CH, Kim W, Jung KH, Park SR, Choi EK, Kim SW, Park KS, Lee DH (2022) Integrin alphavbeta3 Induces HSP90 Inhibitor Resistance via FAK Activation in KRAS-Mutant Non-Small Cell Lung Cancer. *Cancer Res Treat* 54: 767-781 doi:10.4143/crt.2021.651
 31. Choi S, Whitman MA, Shimpi AA, Sempertegui ND, Chiou AE, Druso JE, Verma A, Lux SC, Cheng Z, Paszek M, Elemento O, Estroff LA, Fischbach C (2023) Bone-matrix mineralization dampens integrin-mediated mechanosignalling and metastatic progression in breast cancer. *Nat Biomed Eng* 7: 1455-1472 doi:10.1038/s41551-023-01077-3
 32. Hsu TY, Simon LM, Neill NJ, Marcotte R, Sayad A, Bland CS, Echeverria GV, Sun T, Kurley SJ, Tyagi S, Karlin KL, Dominguez-Vidana R, Hartman JD, Renwick A, Scorsone K, Bernardi RJ, Skinner SO, Jain A, Orellana M, Lagiseti C, Golding I, Jung SY, Neilson JR, Zhang XH, Cooper TA, Webb TR, Neel BG, Shaw CA, Westbrook TF (2015) The spliceosome is a therapeutic vulnerability in MYC-driven cancer. *Nature* 525: 384-388 doi:10.1038/nature14985
 33. Liu L, Ulbrich J, Muller J, Wustefeld T, Aeberhard L, Kress TR, Muthalagu N, Rycak L, Rudalska R, Moll R, Kempa S, Zender L, Eilers M, Murphy DJ (2012) Deregulated MYC expression induces dependence upon AMPK-related kinase 5. *Nature* 483: 608-612 doi:10.1038/nature10927
 34. Zhang Y, Xia F, Liu X, Yu Z, Xie L, Liu L, Chen C, Jiang H, Hao X, He X, Zhang F, Gu H, Zhu J, Bai H, Zhang CC, Chen GQ, Zheng J (2018) JAM3 maintains leukemia-initiating cell self-renewal through LRP5/AKT/beta-catenin/CCND1 signaling. *J Clin Invest* 128: 1737-1751 doi:10.1172/JCI93198
 35. Kaminska B, Wesolowska A, Danilkiewicz M (2005) TGF beta signaling and its role in tumor pathogenesis. *Acta Biochim Pol* 52: 329-337
 36. Babyshkina N, Dronova T, Erdyneeva D, Gervais P, Cherdyntseva N (2021) Role of TGF-beta signaling in the mechanisms of tamoxifen resistance. *Cytokine Growth Factor Rev* 62: 62-69 doi:10.1016/j.cytogfr.2021.09.005