ANTICANCER RESEARCH *36*: xxx-xxx (2016)

No: 5539-Z
Please mark the appropriate section for this paper
☑ Experimental
☐ Clinical
☐ Epidemiological

ADAM29 Expression in Human Breast Cancer and Its Effects on Breast Cancer Cells *In Vitro*

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Abstract. *Background: A Disintegrin And Metalloprotease domain 29 (ADAM29) is involved in many important physiological processes. Recent studies have demonstrated that ADAM29 is a susceptibility locus showing traits as a risk factor for breast cancer under genome-wide significance, however, the clinical relevance and cellular function of ADAM29 in breast cancer have not been reported. Materials and Methods: In this study, we assessed the expression levels of ADAM29 in a cohort of human breast cancer specimens. We also used MDA-MB-231 cells with differing ADAM29 expression and assessed the influence of ADAM29 and its mutations on the MDA-MB-231 cell line. Results: Increased transcript expression of ADAM29 was observed in breast cancer tissues compared to normal ones. The expression of ADAM29 and its mutations in different domains significantly influenced proliferation, migration and invasion of breast cancer cells in vitro. Conclusion: ADAM29 may represent a prognostic factor in human breast cancer, as well as a novel molecular candidate to be used as a therapeutic target.*

Breast cancer is the most common cancer among females according to 2012 GLOBOCAN statistics, nearly 1.7 million women were diagnosed with breast cancer with 522,000 related deaths (1). If breast cancer is detected at stage IIIB or later, the 5-year survival rate may be significant reduced than the one if detected at earlier stages (2). Therefore, it is of high scientific importance to discover an ideal molecular target for diagnosis and treatment of breast cancer.

ADAMs (a disintegrin and metalloprotease) are a family of integral membrane and secreted glycoproteins mediating cell-cell and cell-matrix interaction. They have functional similarity to MMPs in their zinc-binding metalloprotease domains, and are also referred to as the disintegrin, cysteine-rich (MDC) family. They are membrane-anchored glycoproteins containing both protease and adhesion domains that have several biological functions, like encompassing cell adhesion through their cysteine-rich domains that bind to syndecans and fibronectin (3, 4), cell fusion and signaling (5, 6). Studies have shown that several members of the ADAM family are highly expressed in a variety of human carcinomas, likely contributing to tumor development and/or progression (7-9).

A novel human ADAM gene *ADAM29*, located in human chromosome 4q34 (10), was suggested to be involved in cancer development and progression. High *ADAM29* expression was predictive of a long treatment-free interval in Binet stage A B-cell chronic lymphocytic leukemia patients (11). *ADAM29* up-regulation increased proliferation of gastric carcinoma MKN45 cells, whereas cell-cell aggregation was decreased (12). Recently, several genome-wide association studies have shown that *ADAM29* is a susceptibility locus and a risk factor for breast cancer at the genome-wide significance level (13-15). Although *ADAM29* has been considered as playing important roles in different kinds of cancers, the precise role and mechanism of *ADAM29* in breast cancer remain largely unknown.

Moreover, the presence of distinct somatic mutations in several members of the ADAM family suggests a direct

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Key Words: ADAM family, ADAM29, breast cancer mutation.

involvement of these genes in cancer genesis. According to the existing research, *ADAM29* was found with a highest rate of mutation in malignant tumors including melanoma (16), esophageal cancer (17), colorectal cancer (18) and breast cancer (14).

Functional analysis of the frequently mutated *ADAM29* in melanoma demonstrated that the mutations affected adhesion of melanoma cells to specific extracellular matrix proteins and in some cases increased their migration ability (16). However, the effects of *ADAM29* mutations in breast cancer have not been reported.

In this article, the clinical relevance of *ADAM29* expression was analyzed in a cohort of breast cancer samples. The effects of wild-type and point-mutations (P31L, H63Y and L225I) of *ADAM29* on cell proliferation, migration and invasion of MDA-MB-231 were first examined *in vitro*.

Table I. *Patients' characteristics*.

Clinical data	Grouping	N
Tissue sample	Normal	31
	Tumor	111
Tumor grade	1	20
	2	37
	3	52
TNM staging	I	59
	II	35
	III	7
	IV	4
Clinical outcome	Disease-free	80
	With metastasis	7
	With local recurrence	5
	Died of breast cancer	13

Materials and Methods

Human breast specimens. A total of 142 breast samples were obtained from breast cancer patients (31 were background normal breast tissue and 111 were breast cancer tissue). These tissues were collected immediately after mastectomy, and snap-frozen in liquid nitrogen, with the approval of the local research ethical committee. Adjacent background normal mammary tissues were obtained from the dissected mammary tissues. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumor deposits. The median follow-up for the cohort was 120 months (June 2004). The relevant information is provided in Table I.

RNA preparation and real-time quantitative polymerase chain reaction (QPCR). Real time quantitative PCR (QPCR) was performed on the Icycler IQ5 system (Bio-Rad, Hammel Hemstead, UK) to quantify the level of *ADAM29* transcripts in the breast cancer specimens (shown as copies/μl from internal standard). The QPCR technique utilized the Amplifluor system™ (Intergen Inc., England, UK) and QPCR master mix (BioRad, Camberley, UK). Pairs of primers were designed using Beacon Design software (PREMIER Biosoft, Palo Alto, CA, USA): *ADAM29* QPCR primers as follow: sense: 5'-GAATC CCTGG TTTCC CTCAG-3'; antisense: 5'-ACTGAACCTGACCGTACAGTTTCTCCTCACTG TCCAT CTTG-3'. Z-sequence on Q-PCR primers is 5'ACTGAACC TGACCGTACA'3, which is complementary to the universal Z probe (TCS Biologicals Ltd., Oxford, UK). Real-time QPCR conditions were 95˚C for 15 min, followed by 60 cycles at 95˚C for 20 s, 55˚C for 30 s and 72˚C for 20 s.

Immunohistochemical staining of breast specimens. The frozen paired tissues were cut into 7-μm sections, and immunohistochemically stained as described previously (19).

ADAM29 monoclonal antibody was from Abnova (Taiwan, China). A peroxidase conjugated goat anti-mouse immunoglobulin IgG was obtained from ZSGB Biotechnology (Beijing, China). The diaminobenzidine (DAB) chromagen (Cell Signal Technology, Danvers, MA, USA) was used for the colouration. Finally, the sections were observed under the microscope (BX43, Olympus, Tokyo, Japan). Staining was independently assessed by the authors.

Cell line and culture. The breast cancer cell line, MDA-MB-231 was purchased from the Cell Culture Center (cell resource center, shanghai institute for biological sciences, Chinese Academy of Sciences, Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin (Gibco BRC, Paisley, Scotland) in an incubator at 37˚C, 5% CO_2, and 95% humidity.

Preparation of plasmids. ADAM29 cDNA were cloned into GV141 vector by Genechemcompany (Shanghai, China) to obtain the Flag-*ADAM29* expression construct. The constructs of *ADAM29* mutants P31L, H63Y, L225I were generated based on the wide-type plasmid using phusion PCR for site-directed mutagenesis by Genechem company (Shanghai, China). The shRNA targeted to *ADAM29* and the control shRNA plasmids were constructed by Genechem company (Shanghai, China) using vector GV112. The shRNA sequence was 5'-CCGGGCACTCTGACTGATGGTTCTACTCGA GTAGAACCATCAGTCAGAGTGCTTTTTG-3', and 5'-AATTCA AAAAGCACTCTGACTGATGGTTCTACTCGAGTAGAACCATC AGTCAGAGTGC-3'.

Cell transfection. Sub-confluent cells were transfected with various constructs by using lipofectamine reagent according to manufacturer's instructions (Invitrogen, Camarillo, CA, USA). MDA-MD-231 cells were selected in the culture medium with 1,200 μg/ml G418 or 0.5 μg/ml puromycin respectively for further 21 days to generate stable *ADAM29*-expressing cells or *ADAM29* knockdown cells. *ADAM29* expression was verified using western blotting.

Cell proliferation assay. Cell viability was evaluated by a non-radioactive cell counting kit (CCK-8, Dojindo, Kamimashiki-gun, Kumamoto, Japan) assay that was performed according to manufacturer's instructions. Cells were seeded in a 96-well plate at 3×10^3 cells per well, and after every 24 h, add CCK-8 reagent to each well and the plates were incubated for an additional 1 h at 37˚C. Cell viability was measured as the absorbance at 450 nm with an Elx800™ spectrophotometer (BioTek, Winooski, VT, USA).

Table II. *Correlation of ADAM29 mRNA expression and clinical parameters.*

Category	No.	Median	IQR	*p*-Value
T/N				
Normal	31	16	<0.000001-2608	
Tumor	111	390	<0.000001-7863	0.22
Tumor grade				
1	20	26	<0.000001-2695	
2	37	190	<0.000001-41920	0.73
3	52	1365	3-7808	0.10
23	89	619	<0.000001-15645	0.23
TNM staging				
I	59	97	<0.000001-4419	
II	35	1296	2-30063	0.17
III	7	6794	468-30542	0.11
II&III&IV	46	1178	6-28493	0.08
Clinical outcome				
Disease-free	80	404	<0.000001-11190	
With metastasis	7	1060	318-5005	0.62
With local recurrence	5	<0.000001	<0.000001-34.5	0.30
Died of breast cancer	13	1691	60-50553	0.31
Met+dead	20	1375	169-24921	0.29
Met+rec+dead	25	724	<0.000001-6531	0.96

Wound-healing assay. Migratory property of the breast cancer cells was assessed by wound-healing assay, as described previously (20). Images of the wound were recorded under a phase contrast microscope at different time (0 h, 6 h, 12 h, 24 h). Wound closure/cell migration was evaluated with Image-Pro plus.

In vitro Matrigel invasion assay. 2×10^4 cells in 200 μl of culture medium with 1% FBS were added to the upper chamber of transwell chamber (Corning, ME, USA) containing 8.0 μm pores, while the lower chamber was filled with 600 μl of culture medium with 20% FBS. After incubated for 48h, the cells were fixed, stained, and quantified as described before (21).

Statistical analysis. The results of *in vitro* assays were assessed using non-paired (two-sided) Student's *t*-test, one-way ANOVA test with SPSS 19.0 software. *ADAM29* mRNA values obtained in the QPCR study are given as mean transcript copy number per 50 ng of RNA±SD. The relationship between the expression of *ADAM29* and tumor grade, TNM staging and survival status were respectively analyzed using Mann–Whitney *U*-test (Table II). A p-value <0.05 was defined as statistically significant.

Results

Expression of ADAM29 in the clinical breast cohort. *ADAM29* expressed in the cytoplasm of both normal breast epithelial cells and cancerous cells in the invasive ductal carcinoma (Figure 1). The staining appeared to be weaker from the normal breast cells compared with breast cancer cells (Figure 1). *ADAM29* transcript levels were also examined in the breast cohort using quantitative PCR. An increased level of *ADAM29* expression was revealed in breast tumors, compared with normal tissues (Table II). The expression levels of *ADAM29* were found to be elevated in the moderately differentiated (grade 2) and poorly differentiated (grade 3) tumors when compared to well differentiated (grade 1) tumors. A trend was also observed that higher levels of *ADAM29* transcripts tended to be more frequently observed in the local advanced tumors (TNM II and III) tumour. Further analysis of *ADAM29* transcript levels against the clinical aspects demonstrated that increased *ADAM29* expression was correlated with poor disease prognosis (included with metastasis, with local recurrence and died of breast cancer) over the 10 year follow-up period in comparison with those who remained disease-free. These results suggested *ADAM29* expression level is associated with the poorer-outcome patients of breast cancer, though these differences were not statistically significant.

ADAM29 expression affected malignant phenotypes of MDA-MB-231 cells. ADAM29 protein was strongly present in the *ADAM29*-overexpressing cells (Figure 2A) and reduced in the *ADAM29*-knockdown cells (Figure 2B). Compared to control cells, cell proliferation were significantly promoted from the second day, and by up to 54% in cells overexpressing *ADAM29* on day 4 (Figure 2C), while cell proliferation was reduced to 68% on day 4 in MDA-MD-231 *ADAM29*-kd cells (Figure 2D). Cell migration and invasion of *ADAM29* overexpression cells were also increased up to 94% and 20% at 24 h and 48 h, respectively (Figure 2E and G). The knockdown of *ADAM29*

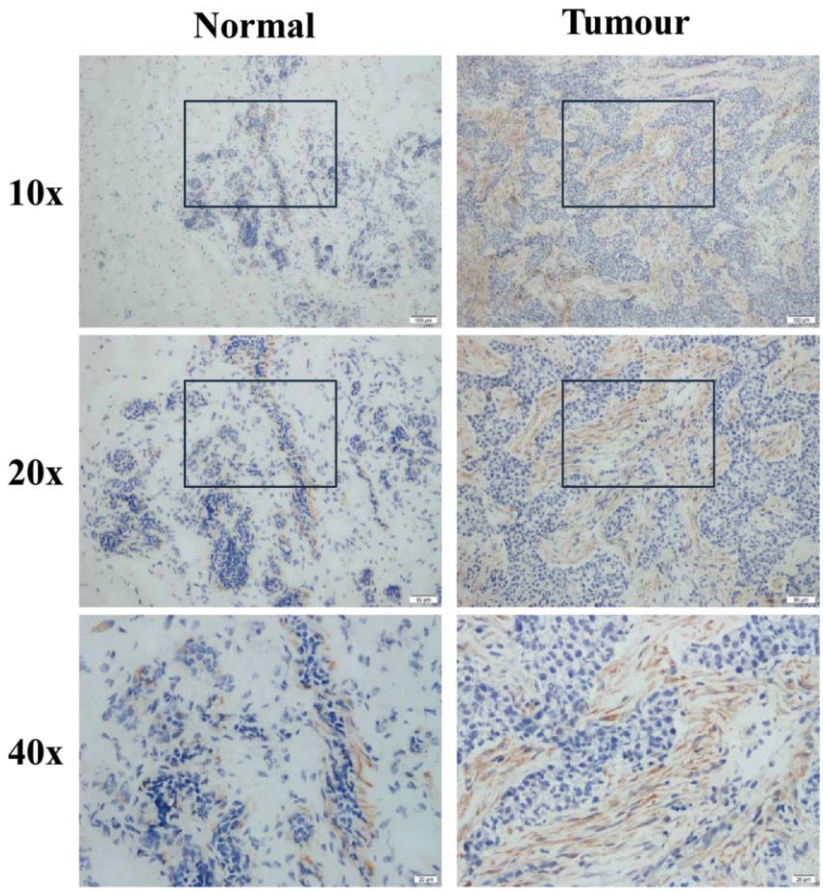

Figure 1. *Expression of ADAM29 in normal breast tissue and breast cancer tissue. Different levels of ADAM29 were detected in the cytoplasm of both normal breast cells and cancerous cells. Immunohistochemical staining revealed the increased staining of ADAM29 in breast cancer sections compared with normal tissue.*

significantly reduced the migratory and invasive nature of breast cancer cells (Figure 2F and H). These results strongly suggested that *ADAM29* play an important role in the regulation of malignant phenotypes of MDA-MB-231 cells by promoting cell proliferation, migration and invasion.

Effects of ADAM29 mutation on cell growth, migration and invasion. ADAM29 was reported to be frequently mutated in melanoma (16). Most mutations were found located in the pro-protein and reprolysin domain of *ADAM29* proteins. To further examine if these mutations could regulate the oncogenic function of *ADAM29*, two missense mutations P31L and H63Y located within proprotein of *ADAM29* and mutation L225I within reprolysin of *ADAM29* were introduced into MDA-MB-231 cells respectively. As demonstrated by western Blotting, these mutants were expressed at similar levels to wild-type *ADAM29* (ADAM29-ex) (Figure 3A). We then explored their effects on the malignant phenotypes of cancer cells. As shown in Figure 3B, each of the *ADAM29* mutations significantly increased the cell proliferation. *ADAM29* P31L and H63Y mutations significantly increased the cell proliferation to a similar level that was seen in wild-type *ADAM29* overexpression cells. While the growth rate of cells overexpressing *ADAM29* L225I was 17.2% more than those expressing wild-type *ADAM29* on day 4, which suggested the stronger capability of promoting cell growth of *ADAM29* L225I. As well as the effects on cell growth, P31L, H63Yand L225I mutations of *ADAM29* increased the capability of cell invasion compared with wild-type *ADAM29* (Figure 3C). Furthermore, three of the *ADAM29* mutants showed the similar effect with the wild-type protein on cell invasion. In contrast, the various ADAM29 mutants reduced cell migration compared to wild-type *ADAM29*, though the more migratory rate was observed in the mutation cells than in vehicle control cells (Figure 3D). These results strongly suggested *ADAM29* and the mutants participated in the regulation of growth, migration and invasion of breast cancer cells.

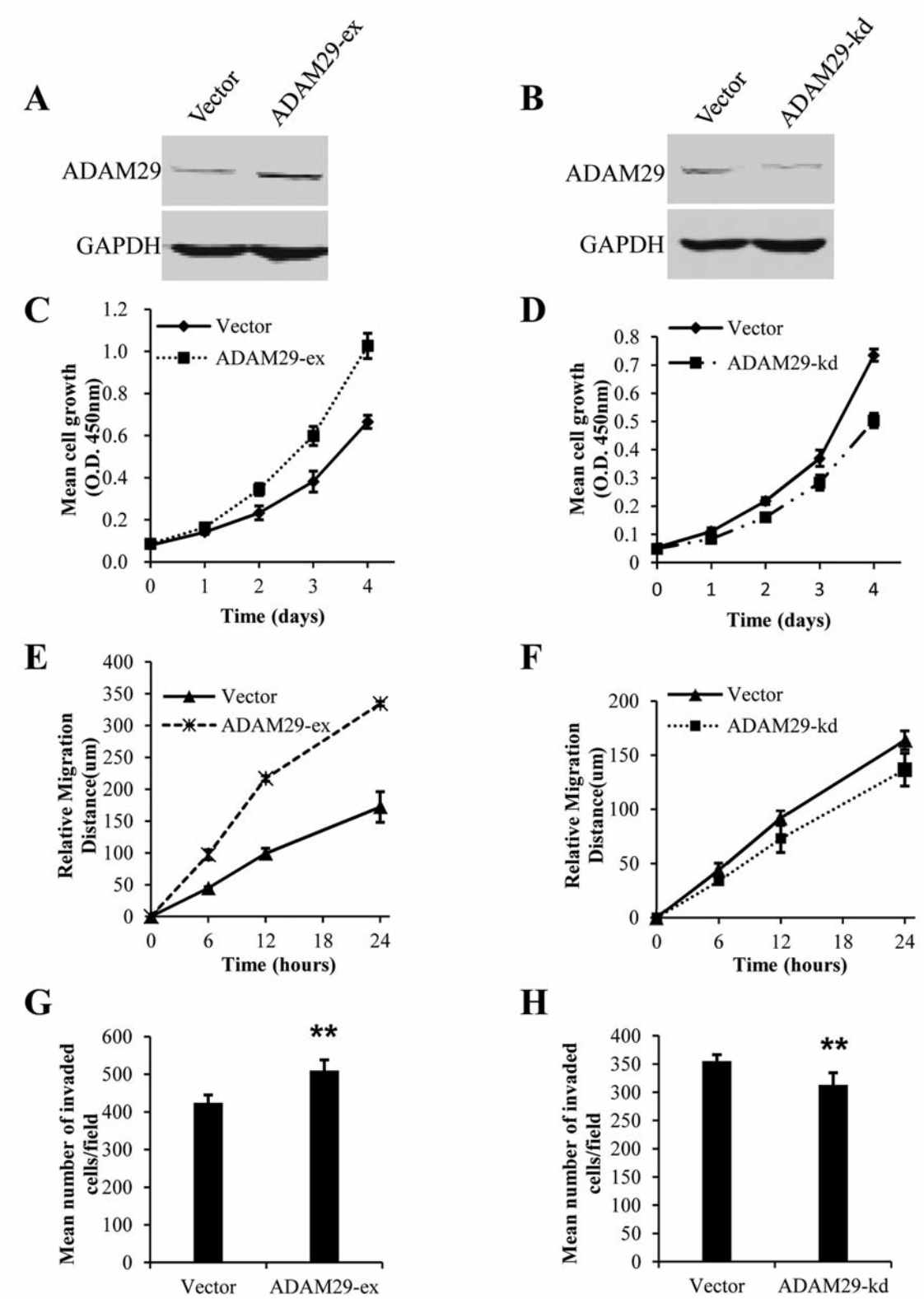

Figure 2. *ADAM29 promotes cell growth, migration and invasion in vitro. A, B: Overexpression and knockdown of ADAM29 at the protein level in breast cancer cell MDA-MB-231 were verified using western blot. C: ADAM29 overexpression promoted MDA-MB-231 cells growth over the experimental time points compared to control plasmid cells. D: Reduced growth rate of cells was seen in ADAM29 knockdown MDA-MB-231 cells. E, G: Significant increase in cell migration and cell invasion were seen in ADAM29 overexpressing cells. F, H: A reduced migration and cell invasion was seen in the MDA-MB-231 ADAM29 knockdown cells. *p<0.05, **p<0.01.*

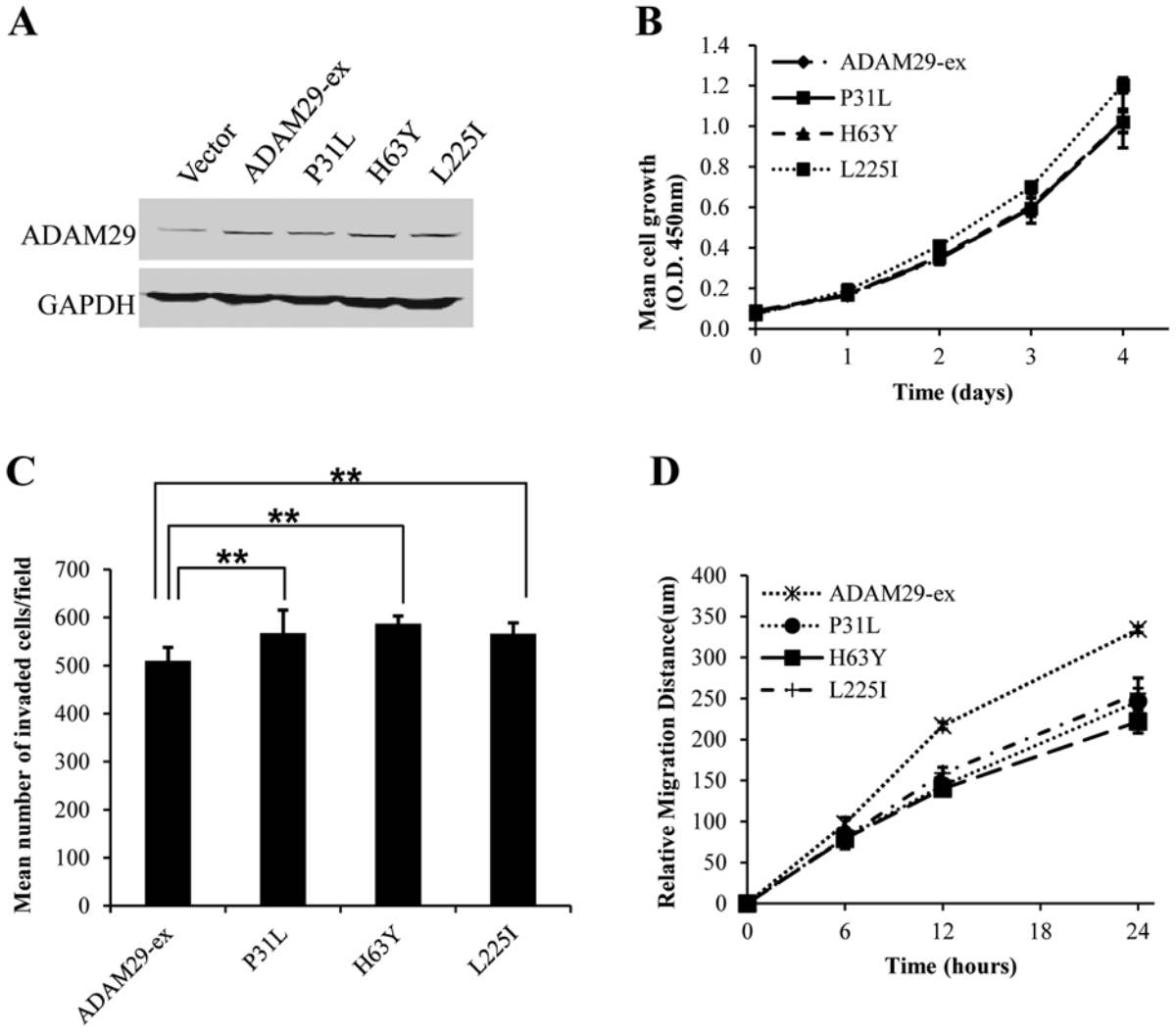

Figure 3. *Effects of mutant ADAM29 on cell growth, migration and invasion. A: The wild-type (ADAM29-ex) and mutants of ADAM29 (P31L, H63Y, L225I) were overexpressed in MDA-MB-231 cells. B: ADAM29 wild-type and mutants promoted the growth of MDA-MB-231 cells at the same level. C: Overexpression of ADAM29 mutations increased the invasive capability of MDA-MB-231 cells. D: The ADAM29 mutants reduced cell migration compared with wild-type ADAM29. *p<0.05, **p<0.01.*

Discussion

Recently, the ADAM family was reported to have association with the progression of human breast cancer (22-25). In the current study, we reported that increased transcript expression of *ADAM29* was observed in the breast cancer tissue sections compared to normal tissues. The follow-up analysis presented that *ADAM29* expression was increased in poorer-outcome patients with breast cancer. The results suggested an oncogenic role of *ADAM29* in breast cancer, which was further confirmed by the *in vitro* function assays. Overexpression of *ADAM29* promoted cell proliferation of MDA-MD-231 cells, and knockdown of *ADAM29* showed the opposite effect on cell proliferation. It was also observed that knockdown *ADAM29* reduced the ability of migration and invasion in breast cancer cell lines. On the contrary, higher expression of *ADAM29* sharply promoted migration and invasion compared with the control cells. The results of both gain-of-function and loss-of-function studies indicated that *ADAM29* acted as an oncogene in breast tumor cells.

A systematic mutational analysis of the ADAMs gene family in human cancers discovered two frequently mutated ADAM genes, *ADAM7* and *ADAM29*. 1,465 mutations in *ADAM29* identified from 727 donors were reported by 40 projects (https://dcc.icgc.org/genes/ENSG00000168594).

Most mutations located in the pro-protein and reprolysin domains of *ADAM29* protein. The expression of the *ADAM29* mutants in melanoma substantially increased the melanoma cell adhesion to collagen I and IV compared to cells expressing wild-type *ADAM29* (16). However, the effects of *ADAM29* mutations in breast cancer have not been reported to date. In order to study the effects of *ADAM29* mutations on cell function, three mutants (P31L, H63Y andL225I) were constructed. P31L and H63Y located in the pro-protein domain of *ADAM29*, while L225I located within the reprolysin domain. Results showed that three mutations of *ADAM29* increased the invasive capability of MDA-MB-231 cells that may play pivotal role in the process of tumor progression and metastasis. In addition, the mutation in the reprolysin domain of *ADAM29* enhanced the oncogenic role of wild type ADAM29 on promoting proliferation of MDA-MD-231 cells. In contrast, the *ADAM29* mutants reduced cell migration compared with wild-type *ADAM29*.

The development of normal breast tissue into breast cancer should undergo the stage of atypical hyperplasia, carcinoma *in situ*, and invasive cancer, then metastases occur. Tumor cells should firstly obtain a strong ability of proliferation and invasion at the early stage of tumor development and progression. Migration is the ability of cells to move to another place, or movement in the blood and lymph vessel. The three mutants of *ADAM29* in our study increased the invasion ability of breast cancer cells, and L225I allow the breast cancer cells to obtain a stronger proliferative ability. These results suggested that *ADAM29* mutants could play an important role mainly in the development of breast cancer at the start and early stage.

In conclusion, our data showed higher levels of *ADAM29* were apparent in patients with poorer outcome. The results of both gain-of-function and loss-of-function studies indicate that *ADAM29* may act as an oncogene in tumor cells. Mutations in different domain of *ADAM29* affected proliferation, migration and invasion of breast cancer. All these findings could provide significant clinical applications, including the use of *ADAM29* as a molecular marker in breast cancer diagnosis, as well as an indicator for prognosis. In addition, *ADAM29* may present a novel molecular candidate for therapeutic target in breast cancer, that requires further study to better understand the mechanism of action.

Acknowledgements

This work was supported by National Natural Science Foundation of the People's Republic of China (No. 81572704, 81471229) and the Foundation of Beijing Educational Committee (No. KM2013100 25003). We wish also to thank the Beijing Municipal Science & Technology Commission for their support (No. Z15110000 1615039).

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Received January 18, 2016
Revised February 19, 2016
Accepted February 22, 2016