

Identification of the functional role of AF1Q in the progression of breast cancer

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Abstract A novel highly metastatic MDA-MB-231HM cells, derived from MDA-MB-231, was established in our institute. RT-PCR, real-time PCR and Western blot showed that AF1Q gene was differentially expressed between highly metastatic MDA-MB-231HM cells and its parental MDA-MB-231 cells. However, its molecular mechanisms in breast cancer metastasis remain to be characterized. To investigate the effects of AF1Q on the progression of human breast cancer cells, in the present study, recombinant expression plasmid vectors of the human AF1Q gene was transfected into MDA-MB-231 cells. We demonstrated that AF1Q overexpression enhanced the *in vitro* proliferation and invasive potential of breast cancer cells. Focused microarray analyses showed that 22 genes were differentially expressed between AF1Q transfected cells and its parental counterparts. Integrin $\alpha 3$, accompanied by up-regulation of Ets-1 and MMP-2, significantly enhanced the *in vitro* invasive potential of human breast cancer cells mediated by AF1Q. Estrogen-responsive ring finger protein gene (EFP), also played a role in the enhancement of *in vitro* proliferation of human breast cancer cells mediated by AF1Q, accompanied by down-regulation of 14-3-3 δ . The association was ER α independent. These results were further demonstrated by RNA interference (RNAi)

experiment *in vitro*. In *in vivo* study, we also demonstrated that AF1Q transfected breast cancer cells grew much faster and had more pulmonary metastases than vector-transfected or its parental counterparts. On the contrary, AF1Q knockdown cells grew slower and had less pulmonary metastasis. Similar effects of AF1Q on integrin $\alpha 3$, Ets-1, MMP-2, EFP, and 14-3-3 δ expression observed *in vitro* studies were also found in the *in vivo* study. Taken together, these results provide functional evidences that overexpression of AF1Q leads to a more progression in human breast cancer, at least in part, through regulating the integrin $\alpha 3$, Ets-1, MMP-2, EFP, and 14-3-3 δ expression.

Keywords Breast cancer · Invasion · Metastasis · Proliferation · AF1Q

Introduction

Breast cancer is the most common malignancy affecting women and the second leading cause of cancer death in US and some big cities in China, surpassed only by lung cancer [1]. Metastasis is a major challenge in cancer management and it is critical to determine molecular markers that definitively distinguish tumors of nonmetastatic potential from those with metastatic potential. To identify such markers, a clearer understanding of the metastatic progression in breast cancer is required. It is now widely accepted that the progression of a non-neoplastic cell to a hyperplastic cell and eventually to one that is capable of metastasis requires the stepwise accumulation of many genetic alterations [2].

However, the molecular mechanisms contributing to breast cancer progression are poorly understood. In our previously study, MDA-MB-435HM, a highly pulmonary

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metastatic variant of parental MDA-MB-435 cells, was derived within the presented model system by six cycles of the pulmonary metastasis implantation to the mammary fat pad (MFP) [3]. Recently another novel highly metastatic MDA-MB-231HM cells, derived from MDA-MB-231, was also established and maintained using the same methods. The differences in pulmonary metastatic potential of the genetically related cell lines in the two models make them valuable systems for understanding the molecular mechanisms underlying breast cancer metastasis. With the application of a cDNA microarray of 8000 genes, we identified 60 candidate genes, that were differentially expressed between highly metastatic MDA-MB-435HM cells and its parental counterparts, and the ALL1-fused gene from chromosome 1q (AF1Q) was one of the leading genes [4].

The human AF1q gene is located on chromosome band 1q21 and codes for a 9-kD protein with no similarity to any other known proteins. So far, the function of AF1Q gene remained unclear. Previous studies have suggested that the overexpression of AF1Q gene might play roles in leukaemia, myelodysplastic syndrome and thyroid tumor [5–8]. Our institute for the first time found that AF1Q might be a mediator of metastasis promotion in MDA-MB-435 cells [4]. But there are some doubts whether this gene is involved in the metastasis of other breast cancer cell metastasis and its functional roles in this complex and multistep process.

Materials and methods

Cell lines and animals

Human MDA-MB-231 breast cancer cell line was bought from ATCC. The highly metastatic MDA-MB-231HM cell line was established by our institute. MDA-MB-231 cells were routinely maintained in Leibovitz's L-15 medium with 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂. The medium was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every 2–3 days, and cells were subcultured by treatment with 0.25% trypsin/0.53 mM EDTA solution. Female athymic BALB/c-*nu/nu* mice, 4–6 wk old, were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China), and housed in laminar-flow cabinets under specific pathogen-free conditions with food and water ad libitum. All experiments on mice were conducted in accordance with the guidelines of National Institutes of Health (NIH) for the Care and Use of Laboratory Animals. The study protocol was also

approved by Shanghai Medical Experimental Animal Care Committee.

Plasmids

The recombinant human AF1Q expression vector pcDNA3.1-AF1Q was constructed and stored in TE buffer (10 mM Tris, pH8.0/1 mM EDTA) at –30°C by our institute.

RT-PCR and Western blotting analysis

Total RNA was extracted from cultured cells using TRIzol[®] Reagent (Invitrogen, San Diego, CA), and then quantitated by spectrophotometry at 260 nm. RT-PCR was performed according to the manufacturer's instructions. (MBI Fermentas, Vilnius, Lithuania) Gene-specific primers for human AF1Q gene (forward: 5'-ATGAGGGACCCTGTGAGTA-3', reverse: 5'-TTAGAGCAAGTCCATTCGA-3'; 273 bps) were synthesized at Shanghai Sangon Biological Engineering & Technology services Co. (Shanghai, China). The cycling conditions were 94°C for 5 min, followed by 28 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and a final extension of 72°C for 7 min. Negative controls were run in parallel to exclude genomic DNA contamination. The templates for negative controls of RT-PCR were not reverse-transcribed. PCR products were separated on a 1.2% agarose gel and imaged on an Alpha Image 950 documentation system (Alpha Innotech, San Leandro, CA). Densitometry of images was performed using NIH Image version 1.62. All experiments were repeated at least three times, and GAPDH (forward: 5'-GGGAGCCAAAAGGGTCATCATC TC-3', reverse: 5'-CCATGCCAGTGAGCTTCCCGTTC-3'; 353 bp) was chosen as an internal control.

To quantitatively determine the expression level of the AF1Q, the cDNA templates of the cells were amplified by real-time quantitative PCR. The reaction was performed using the DNA Engine Opticon[™] 2 real-time PCR detection system (MJ Research, USA) with SYBR Green I. GAPDH was chosen as internal control. The Opticon 2 apparatus was used to measure the fluorescence of each sample in every cycle at the end of the extension, and the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method was used to allow the quantification of the mRNA of these genes. After PCR, a melting curve was obtained by increasing the temperature from 65 to 95°C with a temperature transition rate of 0.1°C /s. The melting curves of all final PCR products were analyzed. The differences in melting temperature of PCR products were allowed to distinguish genuine products from nonspecific products and primer dimers. To ensure that the correct product was amplified in

the reaction, all samples were also separated on 1.2% agarose gel electrophoresis. All PCR conditions and primers were optimized to produce a single product of the correct size.

Western blotting analysis was performed according to the published method with some modifications [9]. Briefly, Equal amounts of protein (50 µg) from different cells were separated by 10% SDS-PAGE and then incubated with mouse anti-human monoclonal antibodies against AF1Q (Abnova Corporation). Target proteins were detected by enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech) and exposure to Biomax ML film (Eastman Kodak, Rochester, NY). Images were captured by Alpha Image 950 documentation system and analyzed by NIH Image version 1.62. Relative protein in different cell lines was normalized to the signal intensity of β -actin as an internal control.

Stable transfections of AF1Q

Exponentially growing MDA-MB-231 cells were transfected with pcDNA3.1-AF1Q using LipofectamineTM 2000 Transfection Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. The stably transfected colonies were isolated and identified by RT-PCR and Western blotting analysis. In this study, two clones were chosen and named of MDA-MB-231/AF1Q-1 and MDA-MB-231/AF1Q-2. The cells only transfected with pcDNA3.1 vector was named of MDA-MB-231/vector. The transfectants were used before passage 20 in all cases to minimize the impacts of clonal diversification and phenotypic instability. For all functional and biological assays, cells between 70 and 90% confluence were used with viability >95%.

Immunofluorescence staining assays

Immunofluorescence staining assays were performed to analyze the distribution and expression of AF1Q in parental and AF1Q transfectants cells. Cells were cultivated on glass coverslips and incubated with the primary antibodies (mouse anti-human monoclonal antibodies against AF1Q). After washed with 1% BSA-TBST, the cells were incubated with a fluorescein-labeled goat anti-mouse IgG (American Qualex, San Clemente, CA) and stained with 0.5 µg/ml DAPI in TBS for 1 min. Then the coverslips were sealed onto a glass slide using aqueous mounting medium and checked under microscope.

Colony formation assay

The colony formation assay was performed to measure growth promotion by AF1Q gene transfection on cells according to the previously reported protocol [10] with some modifications. Identical numbers of parental cells, empty vector, and AF1Q transfectants cells were seeded in 6-well tissue culture plates to form colonies. After 10 days incubation the number of colonies (≥ 20 cells) was counted within a field at $\times 200$ under a light microscope. For each test, a total of five fields were selected at random, and the numbers were averaged. The assay was repeated at least three independent experiments with 0.5×10^4 , 1×10^4 , and 2×10^4 of the cells seeded, respectively.

Cell proliferation assay

Cell proliferation assays were performed to analyze the proliferation potential of parental, empty vector, and AF1Q transfectants cells by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The cells were harvested and plated in 96-well plates at 1×10^3 per well in 100 µl cell culture medium and maintained at 37°C in a humidified incubator containing 5% CO₂. At the indicated time points, 10 µl of the CCK-8 solution was added into the triplicate wells and incubated for 1.5 h, then absorbance at 450 nm was measured to calculate the numbers of vital cells in each well.

In vitro invasion assays

In vitro invasion assays were performed to analyze the invasive potential of parental MDA-MB-231, MDA-MB-231/vector, and AF1Q transfectants with a Matrigel invasion chamber (Becton Dickinson Labware, Bedford, MA) as described previously with some modifications [11]. Each well insert was coated with 100 µl of a 1:3 dilution of Matrigel in serum-free culture medium. Then, a mixture of 200 µl L15 medium with 10% FBS, 200 µl supernatant of corresponding cell culture, and 200 µl supernatant of NIH/3T3 cell culture was added to the lower chambers as a chemoattractant, and 1×10^5 cells in 250 µl of serum-free L15 medium were added to the top of this Matrigel layer. The cells were incubated at 37°C for 24 h. The cell suspension was aspirated, and excess Matrigel was removed from the filter using a cotton swab. Invasion was assessed by counting the cells that had traveled across the filter and were attached to the bottom side of the filter. Then, the

filters were fixed in 10% formalin and stained with H&E. Cells which had invaded through the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of $\times 200$. Five fields should be counted for each sample.

Focused microarray analysis

For the analysis of the differential expression of multiple genes involved in human metastasis, we used the GEArray Q Series Human Tumor Metastasis Gene Array and Human Cancer Signal Transduction Pathway Finder (SuperArray Bioscience, Frederick, MD). Each GEArray membrane contained 96 specific cDNAs known to be involved in human tumor growth, signal transduction and metastasis. 14 cDNAs is duplicated between two arrays. For every cell line, 5 μ g total RNA was extracted and resuspended in RNase-free water. Biotinylated complementary DNA (cDNA) probes were then generated from RNA isolates using linear polymerase chain amplification. The Nonrad GEArray Ampolabeling kit (SuperArray Inc) was used to generate biotin-labeled probes. The cDNA probes generated by linear amplification of the messenger RNA (mRNA) were denatured, and hybridization was performed according to the manufacturer's protocol (SuperArray Inc.). The relative abundance of a particular transcript was estimated by comparing its signal intensity to the signal

derived from the housekeeping genes. Regulated genes were identified by ratio more than 3.0 or less than 0.33, which indicates at least three-fold up-regulation or 67% reduction.

Real-time PCR analysis

To confirm the above microarray results, the six genes (EFP, IL-2, P53, integrin $\alpha 3$, LAMB2 and TRAF1) identified by microarray analysis were randomly chosen for further validation by real-time quantitative PCR as described previously. The primers were designed using Primer 3 software (Table 1).

RNA interference (RNAi) experiments

To further demonstrate the role for the AF1Q gene in the progression of human breast cancer, we utilized the RNA interference (RNAi) technique to down-regulate the AF1Q gene expression using the BLOCK-iTTM Pol II miR RNAi Expression Vector Kits (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions.

Plasmid construction: The engineered pre-miRNA sequence structure is based on the murine miR-155. The pcDNATM6.2-GW/EmGFP-miR plasmid with spectinomycin resistance gene was used for the cloning of small

Table 1 Primers for PCR and their annealing temperatures

Gene		Primer sequence	Product size (bp)	Annealing ($^{\circ}$ C)
EFP	Up	5'-CTGGTGGGTTTGCTCGTGTT-3'	137	58
	Down	5'-GCTGGAGGCTTGACTGTGCT-3'		
14-3-3 δ	Up	5'-GGCCATGGACATCAGCAAGAA-3'	137	58
	Down	5'-CGAAAGTGGTCTTGGCCAGAG-3'		
IL-2	Up	5'-CTGGAGCATTTACTGCTGGATT-3'	110	58
	Down	5'-GCCTTCTTGGGCATGTAAAAC-3'		
P53	Up	5'-TCAGTCTACCTCCC GCCATAA-3'	231	58
	Down	5'-GTGCAGGCCAACTTGTT CAGT-3'		
Integrin $\alpha 3$	Up	5'-GGAAGGAACAAAGACAGGCAAAC-3'	150	58
	Down	5'-GGTAGTGGTGAGTGAGAAAGTGGC-3'		
LAMB2	Up	5'-GACATTCGCAATCTGGAGGAC-3'	195	58
	Down	5'-TGATGGACAGCAGCAGAGGAG-3'		
TRAF1	Up	5'-CCATCTGTCGCTCTTCATCGT-3'	297	56
	Down	5'-CACCCCTAAGTGTCTGGTCTCCA-3'		
MMP2	Up	5'-CAGGCTCTTCTCCTTTCACAAC-3'	398	55
	Down	5'-AAGCCACGGCTTGGTTTTCCCTC-3'		
MMP9	Up	5'-TGGGCTACGTGACCTATGACAT-3'	150	60
	Down	5'-GCCCAGCCCACCTCCACTCCTC-3'		
Ets-1	Up	5'-GGGTGACGACTTCTTGTTTG-3'	274	57
	Down	5'-GTAAATGGAGTCAACCCAGC-3'		

synthetic oligonucleotides. Three different miR155-based AF1Q (GenBank Accession No: NM_006818) targeting sequences were designed by using Invitrogen's RNAi design algorithm on line. Basic local alignment search tool (BLAST) was used on all miRNA sequences to avoid off-target gene silencing. These sequences are shown in Table 2. Based on the computer analysis, these inserted oligonucleotides would specifically bind to homologous sites of AF1Q mRNA, and thus might interfere with AF1Q expression in cultured cells. In order to identify successful construction of recombinant plasmids, the EmGFP forward sequencing primer (5'-GGCATGGACGAGCTGTACAA-3') and miRNA reverse sequencing primer (5'-CTCTA-GATCAACCACTTTGT-3') were designed to perform PCR. After PCR the plasmids which contained the miRNA insert fragments were verified by DNA sequencing. Finally, the mutants were excluded from this experiment and the right one was purified using QIAGEN Plasmid Maxi (tip 500) Kit according to the manufacturer's instructions.

Transfection of stable miRNA plasmids: The pcDNATM6.2-GW/EmGFP-miR expression vectors containing either the AF1Q miRNA insert (pCMV-AF1Q miRNA-980, pCMV-AF1Q miRNA-1082, or pCMV-AF1Q miRNA-1128) or the pcDNATM6.2-GW/EmGFP-miR-neg control plasmid (Invitrogen, Carlsbad, CA, USA) was transfected into target cells with the Lipofectamine 2000 reagent according to the manufacturer's instruction, respectively. After 48 h of transfection, Western blot and RT-PCR were performed to assess the selectivity of AF1Q knockdown as described above. Successfully transfected cells clones were obtained by a long-term culture in a selection medium containing 4 µg/ml Blasticidin. 3 months later, Western blot and RT-PCR were performed to confirm the knockdown of AF1Q.

In vitro invasion assay: The effects of reduced AF1Q expression on the invasion capacity in breast cancer cells were determined by in vitro invasion assay as described previously. All experiments were performed in triplicate.

RT-PCR and Western blotting analysis

To further explore the molecular mechanisms underlying AF1Q mediated progression of breast cancer by integrin $\alpha 3$ and EFP, expression of several related genes, such as AF1Q, integrin $\alpha 3$, MMP-2, Ets-1, MMP-9, EFP, ER α , and 14-3-3 δ , were investigated in the parent, vector transfected, AF1Q transfected, and AF1Q knockdown cells by RT-PCR, real-time PCR and Western blotting analysis, respectively. The primers were designed using Primer 3 software (Table 1). All experiments were performed in three times.

Table 2 The oligonucleotide sequences of shRNA driven by CMV promoter in pcDNATM6.2-GW/EmGFP-miR (AF1Q, GenBank Accession No: NM_006818)

	Top oligo	Bottom oligo
pCMV-AF1QmiRNA-980 (980–1000 nt)	TGCTGTGTAGGTGGCTGTATCTGACACAGTCTTTGGC CACTGACTGACTGTTCAGATAGCCACCTACA	CCTGTGTAGGTGGCTATCTGACACAGTCAGTGGCCAAAAC TGTTCAGATACAGCCACCTACAC
pCMV-AF1QmiRNA-1082 (1082–1102 nt)	TGCTGTGAAGGTGCTGTACTCAAGGAGTCTTTGGCC ACTGACTGACTCTTGAGCAGCACCTTCA	CCTGTGAAGGTGCTGTCTCAAGGAGTCAAGTGGCCAAAAC TCTTGTAGTACAGCACCTTAC
pCMV-AF1QmiRNA-1128 (1128–1148 nt)	TGCTGTCCAAGTTCGAAGGAGTGGATGGTCTTTGGCCACTG ACTGACCATCCACTTCGAACCTGGA	CCTGTCCAGTTCGAAAGTGGATGGTCAAGTGGCCAAAAC CATCCACTCCTTCGAACCTGGAC

Flow cytometry and proliferation assays

To confirm the roles of EFP in progression of breast cancer by AF1Q, cell proliferation assays and flow cytometry were utilized to investigate the proliferation potential. The control, AF1Q transfected, and AF1Q knockdown cells at logarithmic growth phase were harvested and single-cell suspensions containing 1×10^6 cells were made, respectively. The target cells were treated following the standardized protocol and cell cycle analyses were performed by flow cytometry. Cell proliferation assays was also conducted as described previously.

Tumorigenicity and metastasis assays in nude mice

The tumorigenicity and spontaneously metastatic capability of the different cells were determined using an orthotopic xenograft tumor model in the athymic mice as described previously [12]. Animals were divided into four groups, including the parental, empty vector, AF1Q transfectant, and AF1Q knockdown groups. Each group had eight mice. 2×10^6 cells were injected orthotopically into the exposed axillary MFP of anesthetized athymic mice. The cells in AF1Q knockdown group were inoculated in 6 weeks after transfection. Animals were monitored every 2 days for up to 6 weeks for tumor growth and general health. The rate of primary tumor growth of different cells was determined by plotting the means of two orthogonal diameters of the tumors, measured at 7-day intervals. Animals were killed and autopsied at 6 weeks of post-inoculation. The metastasis deposits of the lung were assessed by macroscopic observation to evaluate the metastatic potential. To confirm the presence of lung metastases, sections were cut at 50 μ m intervals and H&E staining were performed.

Tissue samples harvested for Western blot analysis was snap-frozen in liquid nitrogen. The protein expression of AF1Q, integrin $\alpha 3$, Ets-1, MMP-2, EFP, and 14-3-3 δ in xenograft tumor tissues of different groups was investigated by Western blot.

Statistical analysis

Statistical analysis was performed using the software of Statistical Package for the Social Sciences (SPSS) Version 11.5 for Windows, and $P < 0.05$ was considered as statistically significant.

Results

Expression of AF1Q in MDA-MB-231 and MDA-MB-231HM cells

To confirm the roles of AF1Q, RT-PCR, real-time PCR and Western blot analysis of AF1Q were performed in MDA-MB-231 and MDA-MB-231HM cells. As shown in Fig. 1, analysis of replicate samples from MDA-MB-231HM and MDA-MB-231 cells suggested a close correlation between the expression of AF1Q and the metastatic potential of MDA-MB-231 cells.

Stable transfection and expression analysis

To investigate the effects of AF1Q on the metastatic phenotype of MDA-MB-231 cells, stable AF1Q transfectants was established and named of MDA-MB-231/AF1Q-1 and MDA-MB-231/AF1Q-2. As shown in Fig. 2, RT-PCR, real-time PCR and Western blotting analysis revealed that

Fig. 1 RT-PCR, real-time PCR, and Western blot analysis of AF1Q differentially expressed in MDA-MB-231HM (lane 2) and parental MDA-MB-231 cell line (lane 1). **A** and **B**, Differential expression of AF1Q in both cell lines revealed by RT-PCR (**A**) and quantitative real-time PCR (**B**) analysis. GAPDH is shown as an internal control. (**C**) Representative immunodetection of AF1Q is shown. (**D**) Relative protein expression of AF1Q in different cell lines was normalized to the signal intensity of β -actin

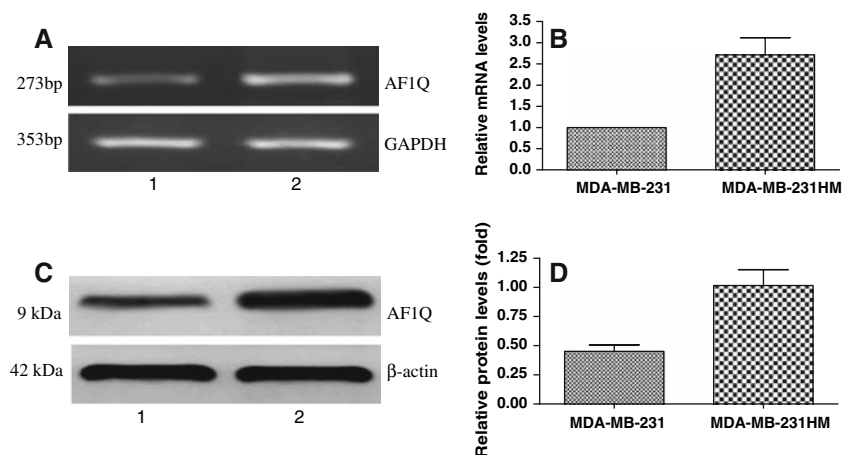
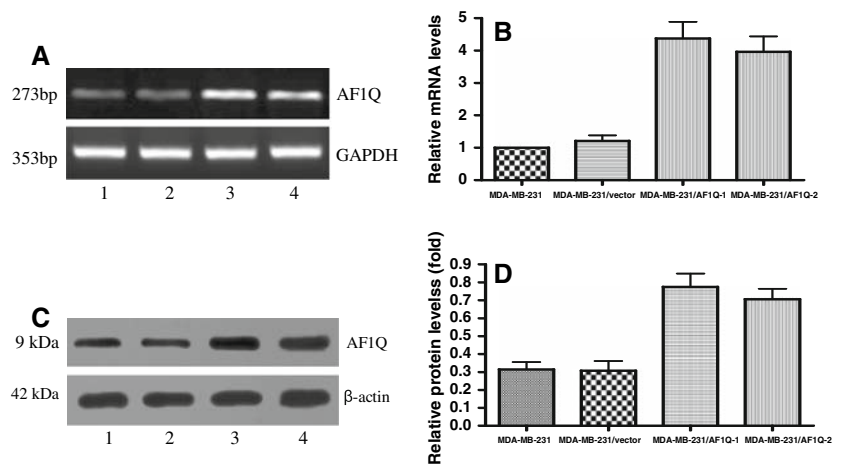


Fig. 2 Expression of AF1Q in AF1Q transfectants and control cells. Lanes 1–4: MDA-MB-231, MDA-MB-231/vector, MDA-MB-231/AF1Q-1 and MDA-MB-231/AF1Q-2. (A) and (B), RT-PCR (A) and quantitative real-time PCR (B) analysis of AF1Q expression in different MDA-MB-231 cells. (C) Western blot analysis of AF1Q expression in different MDA-MB-231 cells. (D) The relative expression of AF1Q protein in different cell lines was normalized to β -actin



levels of AF1Q expression were found much higher in both MDA-MB-231/AF1Q-1 and MDA-MB-231/AF1Q-2 compared with the parental or vector control-transfected cells.

Compared with the parent cells, thicker fluorescence staining for AF1Q expression was observed in transfectant cells

In vitro effects of AF1Q overexpression on MDA-MB-231 cells

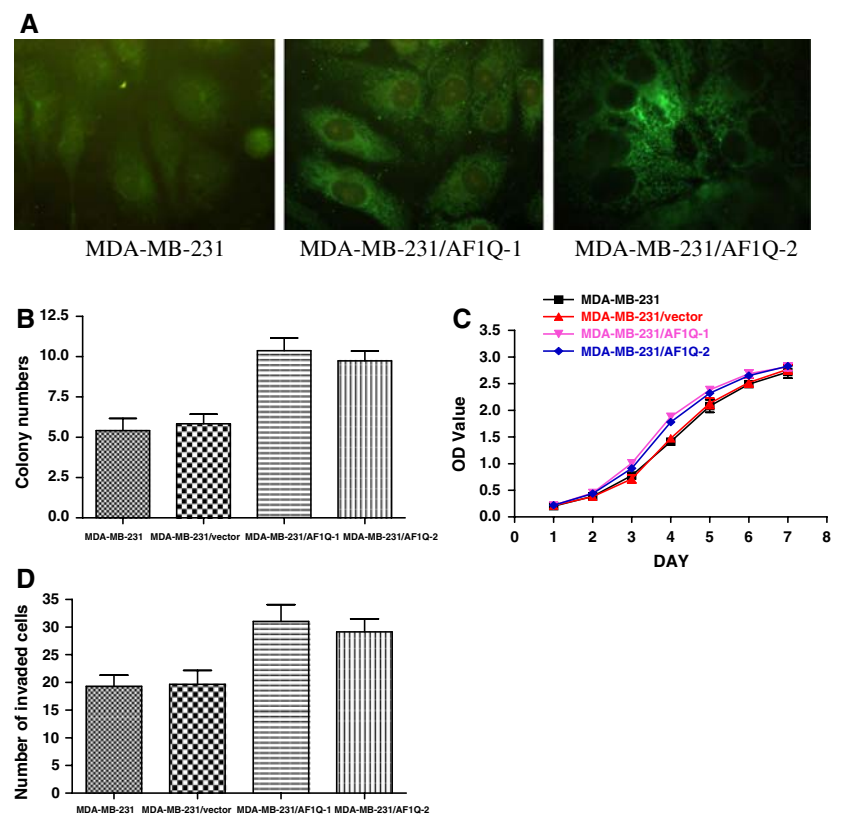
Colony formation assay

Immunofluorescence staining assays

As shown in Fig. 3A, positive fluorescence staining for AF1Q was recognized in the cytoplasm of different cells.

In all of the four groups, recombinant vector-transfected cells formed more colonies than the control, but the statistically significant difference was only observed in the group of 1×10^4 cells (Fig. 3B) ($P < 0.05$).

Fig. 3 In vitro effects of AF1Q overexpression on MDA-MB-231 cells. (A) Representative fluorescent microscopic photographs of MDA-MB-231 transfected with AF1Q. The fluorescence was found to distribute in cytoplasm. Compared with the parent cells, thicker fluorescence staining for AF1Q expression was observed in transfectants cells. (B) Colony formation assay for MDA-MB-231 in the group of 1×10^4 cells. (C) The growth curves of different MDA-MB-231 cells. (D) In vitro matrigel invasion assay, the AF1Q transfected cells were more invasive than its parental counterparts ($P < 0.05$)



Proliferation assay

To investigate whether overexpression of AF1Q could modulate the proliferation rate of MDA-MB-231, we assessed the growth of the parent, AF1Q transfected and vector transfected cells. As shown in Fig. 3C, AF1Q was able to increase the proliferation of MDA-MB-231 cells significantly at 4th and 5th day ($P < 0.05$).

In vitro invasion assays

In vitro invasion assay were performed to determine the effects of AF1Q on cell invasion. The AF1Q transfectants showed higher invasive capacity compared with either parental or vector control-transfected cells (Fig. 3D) ($P < 0.05$).

According to the above results, MDA-MB-231/AF1Q-1 was used as AF1Q transfectant (MDA-MB-231/AF1Q) in the next further study for its higher AF1Q expression and invasive potential.

Focused microarray analysis

To gain insight into the molecular mechanisms associated with the enhanced metastatic potential by AF1Q,

microarray analyses were performed to determine the differences in gene expression profile between the AF1Q transfectant and its parental counterpart. We analyzed the expression of 178 genes known to be involved in human tumor growth, signal transduction and metastasis using this technology. Nine genes were found to be up-regulated and 13 genes were down-regulated (as judged by a >3 -fold change in expression; Table 3).

Real-time PCR confirmation of microarray results

The microarray analyses were validated by real-time PCR. EFP, IL-2, P53, integrin $\alpha 3$, LAMB2 and TRAF1 were randomly chosen to confirm the above microarray results. As shown in Fig. 4, analysis of replicate samples from AF1Q transfectants and the control cells confirmed the level of expression as revealed by microarray analysis. Two of these genes, integrin $\alpha 3$ and EFP, was further investigated the molecular mechanisms associated with the enhanced progression of breast cancer by AF1Q.

RNA interference (RNAi) experiments

After DNA sequencing, three plasmids constructed (pCMV-AF1Q miRNA-980, pCMV-AF1Q miRNA-1082,

Table 3 Differentially expressed genes in AF1Q transfected and its parental MDA-MB-231 cells revealed by focused microarray analysis

GenBank	Description	Ratios
NM_000586	Interleukin 2	0.1467
NM_007294	Breast cancer 1, early onset	0.1844
NM_001202	Bone morphogenetic protein 4	0.1942
NM_002607	Platelet-derived growth factor alpha polypeptide	0.2173
NM_004476	Folate hydrolase (prostate-specific membrane antigen) 1	0.2322
NM_005438	FOS-like antigen 1	0.2378
NM_078626	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.2471
NM_005009	Non-metastatic cells 4, protein expressed in	0.2524
NM_000589	Interleukin 4	0.2561
NM_002423	Matrix metalloproteinase 7 (matrilysin, uterine)	0.2664
NM_002166	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	0.3048
NM_002392	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	0.3080
NM_053056	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	0.3231
NM_002575	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	3.329
NM_005082	Zinc finger protein 147 (estrogen-responsive finger protein)	3.402
NM_000594	Tumor necrosis factor (TNF superfamily, member 2)	3.522
NM_005658	TNF receptor-associated factor 1	4.330
NM_000800	Fibroblast growth factor 1 (acidic)	4.794
NM_000546	Tumor protein p53 (Li-Fraumeni syndrome)	4.881
NM_002293	Laminin, gamma 1 (formerly LAMB2)	5.405
NM_002203	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	6.389
NM_002204	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	7.017

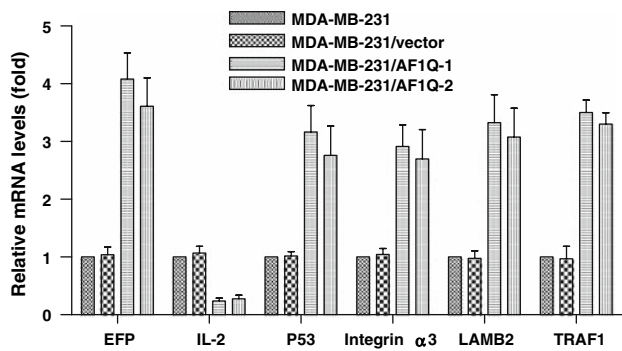


Fig. 4 Real-time quantitative PCR analysis of six selected genes showed differential expression between the AF1Q transfected cells and the control cells. Targets were normalized to reactions performed by using GAPDH

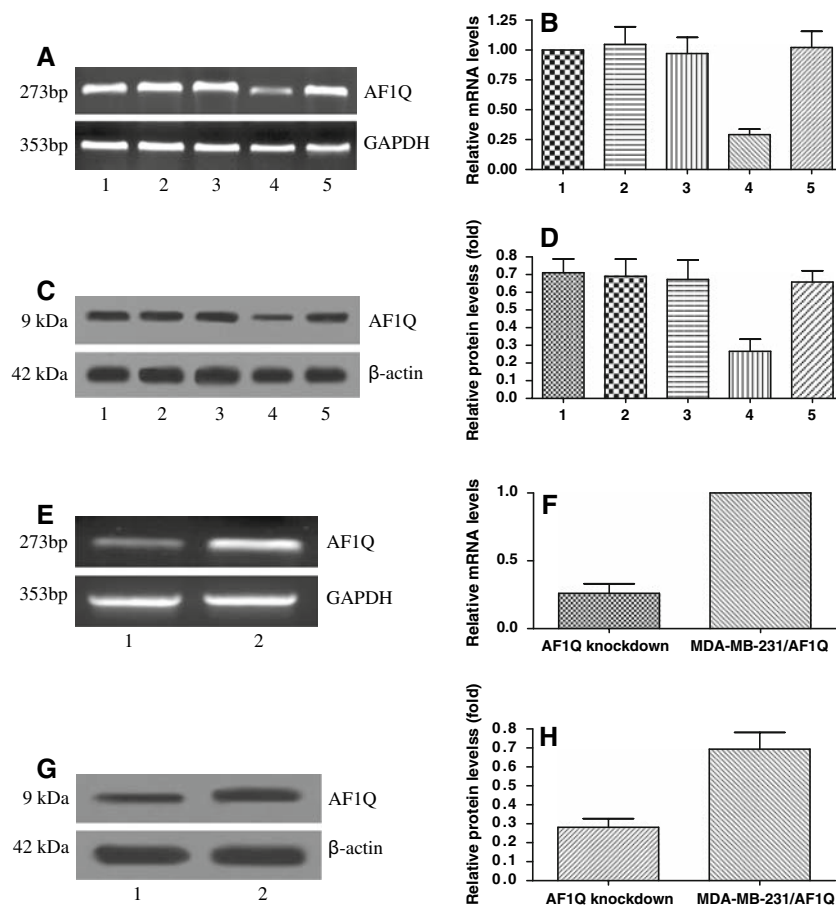


Fig. 5 pCMV-AF1Q miRNA-1128 efficiently silences AF1Q expression in AF1Q transfected breast cancer cells. Lanes 1–5: MDA-MB-231/AF1Q, pCMV-AF1Q miRNA-980 transfected, pCMV-AF1Q miRNA-1082 transfected, pCMV-AF1Q miRNA-1128 transfected, and pCMV-AF1Q miRNA-neg transfected cells. **A** and **B**, RT-PCR (**A**) and real-time PCR (**B**) illustrated that pCMV-AF1Q miRNA-1128, rather than other groups, reduces AF1Q mRNA in MDA-MB-231/AF1Q Cells. **C**, A representative Western blot image illustrated that pCMV-AF1Q miRNA-1128, rather than other groups, reduced

and pCMV-AF1Q miRNA-1128) were transfected into the MDA-MB-231/AF1Q cells. To evaluate inhibition of AF1Q expression, the expression levels of AF1Q were compared among the MDA-MB-231/AF1Q, pCMV-AF1Q miRNA-980 transfected, pCMV-AF1Q miRNA-1082 transfected, pCMV-AF1Q miRNA-1128 transfected and the pCMV-AF1Q miRNA-neg transfected cells at 48 h after transfection. As shown in Fig. 5A–D, AF1Q were strongly inhibited in pCMV-AF1Q miRNA-1128 transfected cells ($P < 0.05$).

In order to assess the phenotype of tumor cells in which AF1Q expression was inhibited over a long-term period, one of clones from pCMV-AF1Q miRNA-1128 transfected

AF1Q protein. **(D)** The relative expression of AF1Q protein in above different cells was normalized to the signal intensity of β -actin. **E** and **F**, RT-PCR (**E**) and real-time PCR (**F**) showed that pCMV-AF1Q miRNA-1128 decreased the AF1Q mRNA in MDA-MB-231/AF1Q cells at 3 months after initial transfection. **(G)** Western blot analysis showed that pCMV-AF1Q miRNA-1128 decreased the AF1Q protein in MDA-MB-231/AF1Q cells at 3 months after initial transfection. **(H)** The relative expression of AF1Q protein in above different MDA-MB-231/AF1Q cells was normalized to the signal intensity of β -actin

cells was chosen and cultured for a long-term period in a selection medium containing Blasticidin. At 3 months, RT-PCR, real-time PCR and Western blotting analysis of the clone cells demonstrated decreased AF1Q expression compared with MDA-MB-231/AF1Q cells (Fig. 5E–H) ($P < 0.05$).

AF1Q knockdown inhibited breast cancer cell invasion in vitro and down-regulation of integrin $\alpha 3$, MMP-2, and Ets-1

The pCMV-AF1Q miRNA-1128 was used to knockdown the AF1Q gene in MDA-MB-231/AF1Q cells. As shown in Fig. 6A, reduced AF1Q expression in MDA-MB-231 cells significantly inhibits the invasive capacity in vitro as compared with the control cells ($P < 0.05$). Integrin $\alpha 3$ is one of up-regulation genes in the AF1Q transfected cells and its expression is related to the invasive potential of cancer cells. Integrin $\alpha 3$ was reported to be implicated in the expression of Ets-1, MMP-2 and MMP-9. RT-PCR, real-time PCR and Western blot analysis showed that AF1Q transfectants constitutively expressed high levels of AF1Q, integrin $\alpha 3$, Ets-1 and MMP-2, and the AF1Q knockdown cells expressed low levels of these genes

(Fig. 6B–E). However, we didn't find the significant differences levels of MMP-9 among the above cells (data not show).

AF1Q mediates the proliferation potential of breast cancer cells by 14-3-3 δ through EFP in vitro

To further explore the molecular mechanisms enhanced proliferation potential of breast cancer by AF1Q in vitro, we focused on EFP gene. As shown in Fig. 7A–D, RT-PCR, real-time PCR and Western blot analysis showed that AF1Q transfectants constitutively expressed high levels of AF1Q and EFP, and the AF1Q knockdown cells expressed low levels of AF1Q and EFP. Western blot analysis also showed the down-regulation of 14-3-3 δ in AF1Q transfectants and up-regulation in AF1Q knockdown cells. However, RT-PCR and real-time PCR did not show any significant differences in 14-3-3 δ mRNA expression between them. The reasons might be that EFP ubiquitinated and degraded the 14-3-3 δ protein by the proteasome. So we could not find the difference expression in mRNA levels.

The proliferation potential mediated by AF1Q was confirmed by cell cycle analysis using flow cytometry and proliferation assays. Flow cytometry analysis showed that a

Fig. 6 Down-regulation of AF1Q in breast cancer cells decreased the invasion capacity in vitro, accompanied with inhibition of integrin $\alpha 3$, MMP-2, and Ets-1. Lanes 1–4: the parents, empty vector, AF1Q transfected, and AF1Q knockdown (MDA-MB-231/AF1Q pCMV-AF1Q miRNA-1128 transfected) cells. **(A)** In vitro invasion assays of the invasive potential of AF1Q knockdown cells and its controls. AF1Q knockdown cells was less invasive than its controls ($P < 0.05$). **(B)** and **(C)**, RT-PCR **(B)** and quantitative real-time PCR **(C)** analysis of AF1Q, integrin $\alpha 3$, MMP-2, and Ets-1 expression is shown. **(D)** Western blot analysis of AF1Q, integrin $\alpha 3$, MMP-2, and Ets-1 expression in different cells. **(E)** The relative expression of AF1Q, integrin $\alpha 3$, MMP-2, and Ets-1 protein in different cell lines was normalized to the signal intensity of β -actin

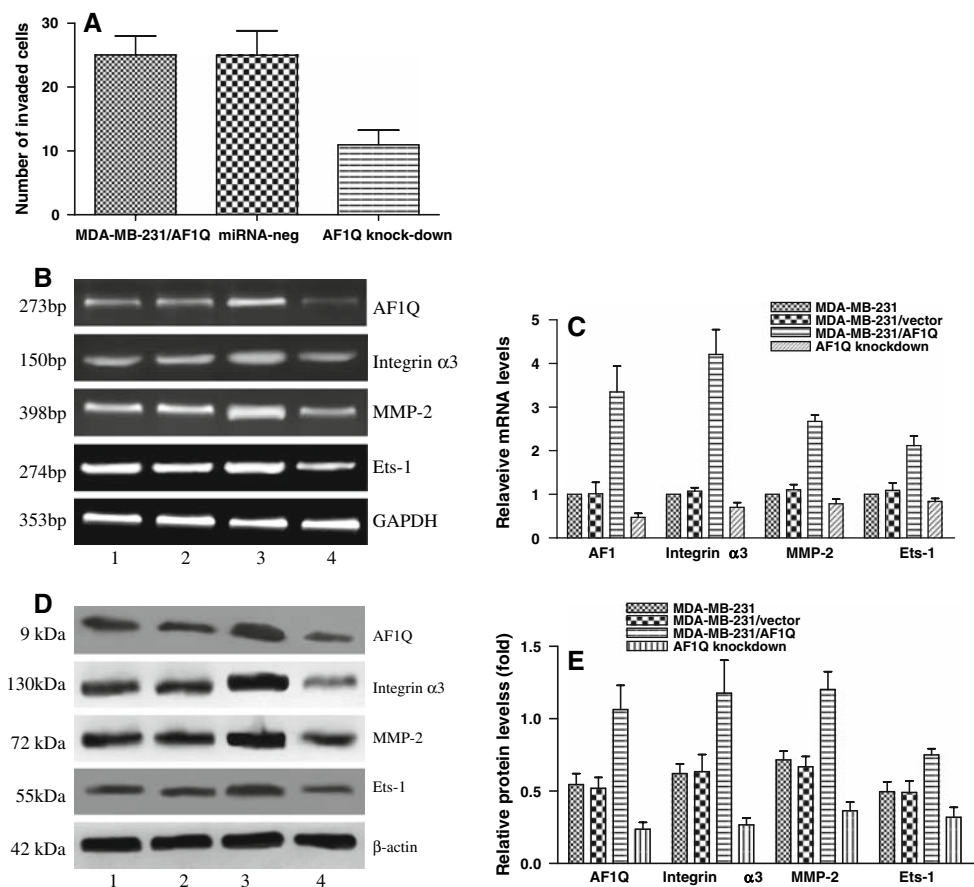
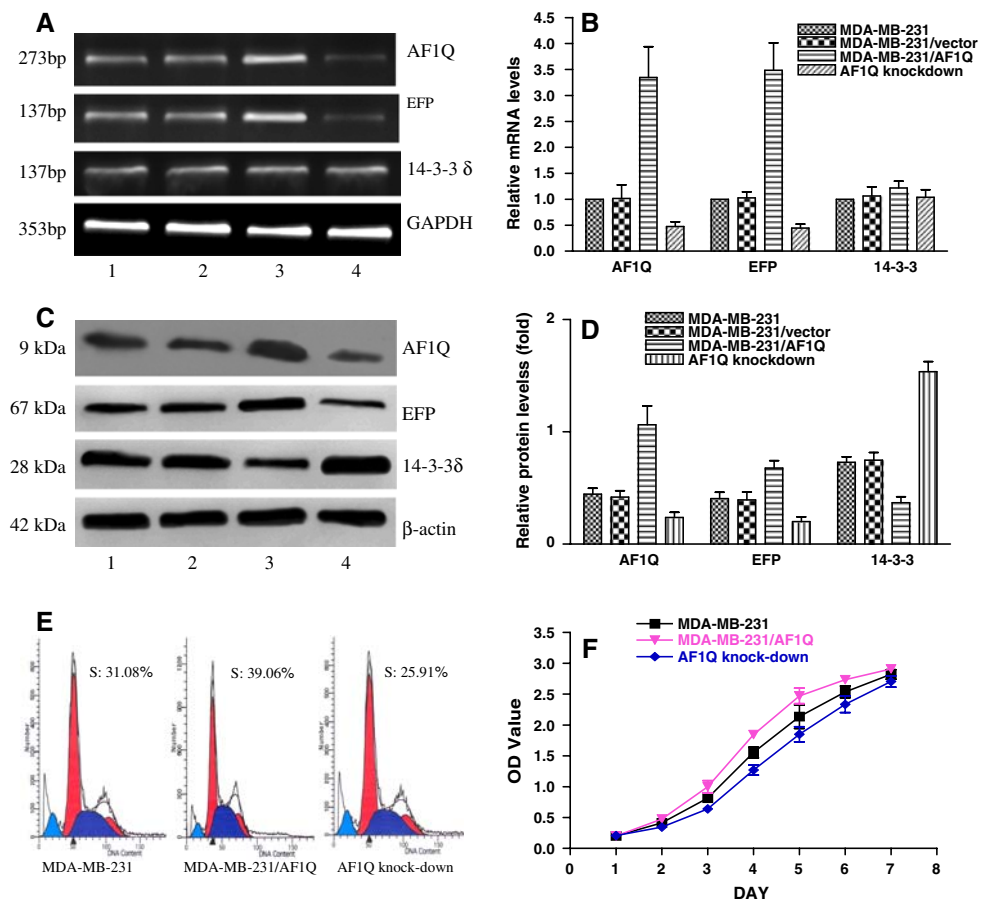


Fig. 7 AF1Q promoted cell progression in vitro, up-regulated EFP and down-regulated 14-3-3 δ protein expression. Lanes 1–4: the parents, empty vector, AF1Q transfected, and AF1Q knockdown cells. **A** and **B**, RT-PCR (**A**) and quantitative real-time PCR (**B**) analysis of AF1Q, EFP, and 14-3-3 δ expression is shown. (**C**) Western blot analysis of AF1Q, EFP, and 14-3-3 δ expression in different cells. (**D**) The relative expression of AF1Q, EFP, and 14-3-3 δ protein in different cell lines was normalized to the signal intensity of β -actin. (**E**) Representative results of flow cytometric analysis. (**F**) The growth curves for the different cells in vitro proliferation assay



high percentage of AF1Q transfected cells and low percentage of AF1Q knockdown cells were in the proliferating stages, particularly in S phase (Fig. 7E). Proliferation assays also confirmed that AF1Q mediated the proliferation potential of breast cancer cells by 14-3-3 δ through EFP in vitro (Fig. 7F).

As we all know, MDA-MB-231 cells is ER α negative and EFP gene is one of the estrogen receptor (ER α) target genes. To explore the difference expression of ER α in the above parent, AF1Q transfected, and AF1Q knockdown cells, RT-PCR, real-time PCR and Western blot analysis were used. However, we couldn't find the any increased levels of ER α in the AF1Q transfected cells (data not show).

Tumor growth and pulmonary metastasis in the athymic mice were regulated by the expression of AF1Q

The effect of AF1Q expression on tumor growth and metastasis was further assayed in an athymic mice model. Results revealed that AF1Q transfected tumor grew faster than either vector-transfected or parental group and AF1Q knockdown group grew slower than the other three groups in

the athymic mice (Fig. 8A). To study pulmonary metastasis, at the experimental endpoint, lungs were examined physically at autopsy and the overt surface metastases were observed. The surface metastases were found in 37.5% of mice in AF1Q transfected group, 12.5% of the parent groups and none of the AF1Q knockdown group. The difference between the AF1Q transfected group and the AF1Q knockdown group was significant. ($P < 0.05$) Furthermore, lungs were subjected to microscopic examination for morphological evidence of tumor cells by light microscopy on H.E-stained paraffin sections. The most micrometastases were observed in AF1Q transfected mice. Only a few micrometastases or no micrometastase were observed in mice of parental, vector-transfected, or knockdown groups (Fig. 8B). In addition, similar effects of AF1Q on integrin α 3, Ets-1, MMP-2, EFP, and 14-3-3 δ expression observed in the in vitro studies were also found in the in vivo studies (Fig. 8C and D).

Discussion

Until recently, the specific mechanisms that actually promote breast cancer metastasis still remain unclear. In the

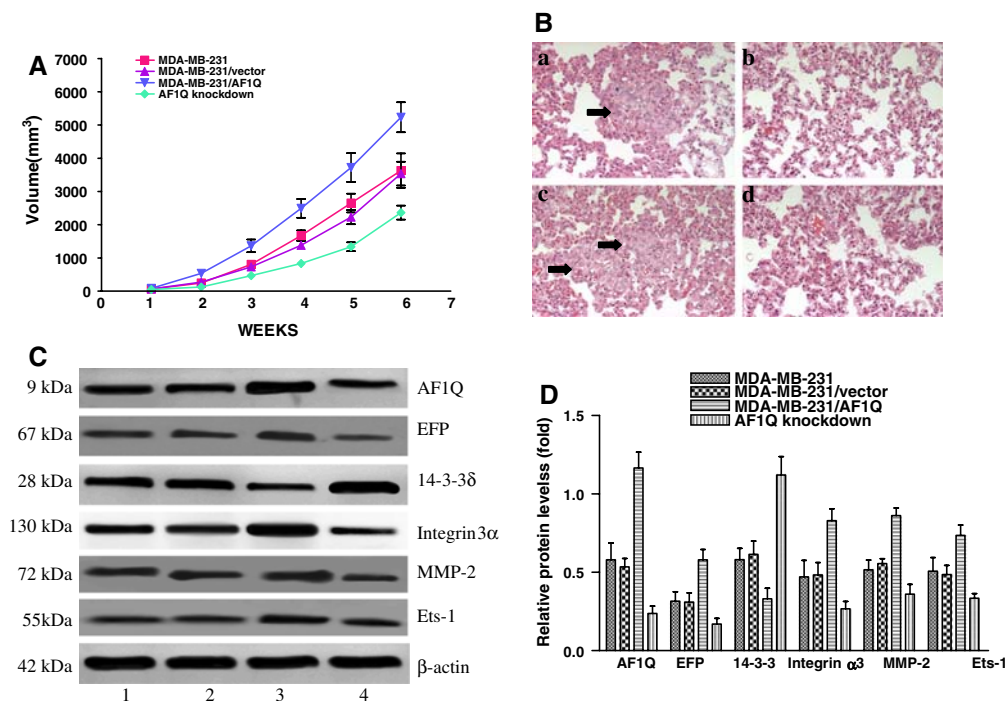


Fig. 8 Regulation of the growth and pulmonary metastasis of MDA-MB-231 cells by AF1Q in vivo studies. **(A)** The growth curves for the parents, empty vector, AF1Q transfected, and AF1Q knockdown cells in in vivo proliferation assay. **(B)** Photomicrographs of micrometastases in lung sections (arrow) obtained from mice bearing the parents (a), vector transfected (b), AF1Q transfected (c), and AF1Q knockdown (d) tumors for MDA-MB-231 cells (H.E, $\times 200$).

previous study, our institute employed the high-throughput cDNA microarray and comparative proteomic system to identify a number of genes and proteins, such as AF1Q, peroxiredoxin 6 and so on, that showed significantly increased expression levels in the highly metastatic MDA-MB-435HM cells compared with its parental counterparts [3, 4].

The AF1q gene was identified as a mixed lineage leukaemia (MLL) fusion partner in an infant with acute myeloid leukaemia (AML) carrying a t(1; 11) (q21; q23) translocation [13]. Tse et al. found that high AF1q expression indicated a poor prognosis in children with AML and its levels might provide a marker for risk stratification in patients with MDS [7]. Jacques et al. reported the AF1Q might be involved in tumorigenesis of thyroid oncogenic tumors [5]. Our institute also demonstrated that AF1Q has a role in metastatic potential of MDA-MB-435 cells. However, the function of AF1q remains to be defined.

In the present study, we also found that AF1Q was up-regulation in MDA-MB-231HM cells and it suggested that AF1Q played a role in the metastatic potential of MDA-MB-231HM cells. We generated stable MDA-MB-231/AF1Q transfectants and tested them for any alterations in

(C) The protein levels of AF1Q, EFP, 14-3-3 δ , integrin $\alpha 3$, MMP-2, and Ets-1 were detected by Western blot in xenografts of different groups. Lanes 1–4: the parents, empty vector, AF1Q transfected, and AF1Q knockdown. **(D)** The relative expressions of AF1Q, EFP, 14-3-3 δ , integrin $\alpha 3$, MMP-2, and Ets-1 protein in above different groups was normalized to the signal intensity of β -actin

the invasive and proliferation potential in vitro. As expected, our results showed that AF1Q also could enhance the invasive and proliferation potential of MDA-MB-231 cells in vitro. Furthermore fluorescence staining for AF1Q was recognized in the cytoplasm, which appears to contradict to published literature regarding AF1Q location. Lin et al. described that intact wild type human AF1Q cDNA encodes a transmembrane protein [14]. Subsequent analysis is needed to explain the disparity in subcellular locations of AF1Q.

Focused microarray analyses were utilized to investigate the molecular mechanisms of AF1Q in breast cancer metastasis. Our results showed that nine genes were found to be up-regulated and integrin $\alpha 3$ is one of up-regulation genes in the AF1Q transfected cells. Integrin $\alpha 3$ belongs to the integrin family members and the integrin family serves as adhesion receptors for extracellular matrix proteins and cellular counterligands. These adhesion receptors are heterodimers of transmembrane glycoproteins (α and β subunits); various combinations of α and β subunits produce polymorphisms of ligand specificity. The expression of integrin $\alpha 3\beta 1$ was demonstrated to be positively correlated with the increased invasiveness of the tumors [15–17]. These studies suggest that integrin $\alpha 3$ is also involved in the

invasion of cancer cells. The mechanisms that integrins modulate cancer cell migration and invasion are not entirely known, but some reports have shown that the integrin is implicated in the production and/or activation of MMP-9 and MMP-2 [18–20]. Kato et al. found that the Ets family of transcription factors regulated the expression of integrin $\alpha 3$ [21]. The Ets transcription factors were also reported to be involved in tumor metastasis through the promotion of angiogenesis and the expression of MMPs [12, 22, 23]. These observations suggest that integrin $\alpha 3$, Ets and MMPs cooperatively promote adhesion and invasion of cancer cells during the metastatic processes.

In the present study, we demonstrated that overexpression of AF1Q could up-regulate the expression of integrin $\alpha 3$, Ets-1, and MMP-2 accompanied with the increased *in vitro* invasive potential in MDA-MB-231 cells. The results were confirmed in AF1Q knockdown cells. However, we could not find any difference expression of MMP-9 levels. More studies should be done to explain the disparity.

In the present study, a miRNA expression vector based on murine miR-155 sequence was used to construct AF1Q targeting RNAi Expression Vector. One construction, chosen from three different miR155-based AF1Q targeting pre-miRNA pol II Vectors, was used to decrease the expression of AF1Q and generate the stable transfectants of AF1Q knockdown in MDA-MB-231 cells. The stable inhibition of AF1Q was observed in three months, which provide the confirmation of its utilization *in vivo*. The ability of invasion was analyzed in AF1Q knockdown breast cancer cells and the obviously decreased *in vitro* invasion was observed. All the results confirmed the roles of AF1Q in the invasive potential of breast cancer.

The estrogen-responsive ring finger protein (EFP) is another up-regulation gene in the AF1Q transfected cells from the results of microarray analysis. The human EFP gene, one of the estrogen receptor (ER) target genes, is located at chromosome 17q23.1 and codes a 67KD protein [24]. It has an estrogen-responsive element (ERE) at the 3'-untranslated region (UTR) and could mediate estrogen-induced cell growth. Some studies [25–27] showed that high expression of EFP was correlated with the cancer development, which was mediated by the down-regulation of 14-3-3 δ . 14-3-3 δ is a p53-inducible protein that sequesters mitotic Cdks in the cytoplasm to inhibit entry into mitosis. To allow cell-cycle progression, estrogens stimulate production of EFP, which binds and ubiquitinates 14-3-3 δ . 14-3-3 δ marked with ubiquitin chains is recognized and degraded by the proteasome. Released Cdks are free to escape into the nucleus and promote mitotic entry.

In the present study, we demonstrated that up-regulation of EFP were accompanied with the inhibition of 14-3-3 δ protein in AF1Q transfected cells. Cell cycle analysis also

showed that EFP overexpression could increase the percentage of cells in the proliferation stage of the cell cycle. However, we could not find any difference expression in 14-3-3 δ mRNA levels between the AF1Q transfected and its control cells. The reasons might be that EFP ubiquitinated and degraded 14-3-3 δ protein by the proteasome.

MDA-MB-231 cell is ER α negative. However, we couldn't find any increased levels of ER α in the AF1Q transfected cells. How was EFP up-regulated? One plausible explanation is there might be some relationship between AF1Q and EFP that is estrogen independence. Urano et al. [28] have found that EFP could promote a switch from estrogen-dependent to estrogen-independent proliferation of breast cancer cells. They demonstrated that estrogen-dependent MCF-7 breast cancer cells forced to express EFP gained the ability to form tumors in nude mice in the absence of estrogen.

In this study, we also demonstrated that AF1Q could enhance the orthotopic tumor growth and pulmonary metastasis *in vivo*. On the contrary, AF1Q knockdown cells showed the decreased ability in breast cancer progression. These changes were associated with regulation of EFP, integrin $\alpha 3$, Ets-1, MMP-2, and 14-3-3 δ protein levels in xenograft tumors. All these results indicate that AF1Q promote breast cancer progression, partially via regulation of EFP, integrin $\alpha 3$, Ets-1, MMP-2, and 14-3-3 δ expression. It might provide a potential therapeutic way for breast cancer development.

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