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Somatic mutational analysis of FAK in breast cancer: A novel gain-of-function mutation due to deletion of exon 33

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ABSTRACT

Focal adhesion kinase (FAK) regulates cell adhesion, migration, proliferation, and survival. We identified a novel splicing mutant, FAK-Del33 (exon 33 deletion, KF437463), in both breast and thyroid cancers through colony sequencing. Considering the low proportion of mutant transcripts in samples, this mutation was detected by TaqMan-MGB probes based qPCR. In total, three in 21 paired breast tissues were identified with the FAK-Del33 mutation, and no mutations were found in the corresponding normal tissues. When introduced into a breast cell line through lentivirus infection, FAK-Del33 regulated cell motility and migration based on a wound healing assay. We demonstrated that the expression of Tyr397 (main auto-phosphorylation of FAK) was strongly increased in FAK-Del33 overexpressed breast tumor cells compared to wild-type following FAK/Src RTK signaling activation. These results suggest a novel and unique role of the FAK-Del33 mutation in FAK/Src signaling in breast cancer with significant implications for metastatic potential.

1. Introduction

Breast cancer is currently the top cancer reported in women worldwide, both in the developed and in the developing world. It is estimated that there were 458,000 breast cancer-related deaths in 2008 worldwide [1]. Metastasis is the major reason for breast cancer-related deaths. Primary tumors in patients most often metastasize to the axillary lymph nodes or to distant organs after surgery or the administration of chemotherapy [2,3]. To improve breast cancer therapy, new potential therapeutic targets are required.

The potential role of RTKs, including FAK and its activation in breast cancer, is of particular interest. Considering that FAK is involved in tumor formation and progression makes FAK a potentially important new therapeutic target [4]. The small molecule inhibitors targets FAK have been developed for use as potential cancer therapies [5]. Many previous studies have shown that elevated FAK expression correlates with breast tumor malignancy and poor prognosis [6–8]. In animal models, the stable knockdown of FAK expression in breast carcinoma cells inhibits breast carcinoma metastasis to lungs, and FAK deletion could block cancer stem/progenitor self-renewal and invasion [9]. In cell models, FAK-deficient breast cancer cells display enhanced assembly and dynamics of invadopodia, which could be rescued by the expression of intact wild-type FAK [10].

FAK sequence alterations, which result from alternative splicing and/or promoter usage, have been characterized. One transcript from this alternative promoter results in the production of a truncated isoform of FAK, which lacks its N-terminal and catalytic domains, termed FRNK (FAK-related non-kinase). FRNK acts as a dominant negative regulator of FAK, which inhibits the proliferation and migration of vascular smooth muscle cells, induces anoikis in neonatal rat ventricular myocytes and inhibits growth-factor mediated signals to MAP kinase in most FAK cells [11,12]. Various alternative splice variants, which predict changes in the amino acid sequence of FAK, were found in the brain [13–15]. These variants include the following: Pro-Trp-Arg, which defines the FAK + isoform; boxes 28, 6 and 7, in reference to the number of amino acids encoded by these exons; and FAK isoforms including boxes 13, 14 and 31 et al. Altogether, these results show that multiple FAK transcripts are expressed in the adult human brain; however, these transcripts appear to be expressed at low levels in humans, which suggests that these alternative transcripts may be side products and/or play a regulatory role in transcription.

We performed mutational analysis of the FAK gene in breast cancer tissues. Novel somatic alternative splicing mutant of FAK

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was identified in breast and thyroid cancer. We developed a Taqman MGB probe-based technology for mutation isolation in 21 paired samples of breast tumor/normal tissues. The analysis shows the mutation occurred in 3 of the 21 tumor samples. FAK-Del33 mutation was demonstrated to have a positive phenotype with increased cell motility and migration along with FAK/Src pathway activation.

2. Materials and methods

2.1. Breast tissue specimens

For the mutation analysis, fresh tissues (paired tumor/normal tissues) from breast cancer patients were collected after surgery and subjected to RNA extraction using the RNA Trizol reagent (Invitrogen; California).

2.2. Plasmids

The human FAK cDNA plasmids pCR2.1-FAK-Wt and pCR2.1-FAK-Del33 were gifts from Dr. Xinmin Zheng (Cornell University).

2.3. RQ-PCR assay developed for FAK-Del33 mutant detection

RQ-PCR shared a common pair of primers and F/control probes, but differed in the F/mut probe. The following probes and primers were designed: the forward primer in exon 32 ($P_{FAK\text{-}F}$); the reverse primer in exon 34 ($P_{FAK\text{-}R}$); F/control probe in exon 32; and F/mut probe in exon 33 (Fig. S1). The qPCR mixture reaction contained 2 μl 10 × Taq Buffer, 1 U Taq (Promega; Wisconsin), 10 nM primers, 10 nM probe, 25 nM dNTP and 1 μl cDNA (1/5 diluted) in a total volume of 20 μl. We used an ABI PRISM 7500 Sequence Detection System (Applied Biosystems; California) for sample amplification and analysis. The amplification conditions were as follows: 3 min at 95 °C, 10 s at 58 °C, 10 s at 72 °C, followed by 40 cycles.

2.4. Cell lines

All human cancer cell lines [MDA-MB-468, MDA-MB-453, and MDA-MB-435s (isolated from a breast cancer patient, yet melanoma derived)] and the HEK293T cells were obtained from the American Type Culture Collection (ATCC) and cultured following ATCC protocols.

2.5. Cloning and expression of FAK-Wt and FAK-Del33 in cell lines

FAK was overexpressed using a pGIPZ lentiviral vector-based expression system (Open Biosystems, Australia). The ORF regions of FAK-Wt and FAK-Del33 were generated from pCR2.1-FAK through PCR amplification. To generate the lentivirus, the pGIPZ-FAK construct and packaging plasmids (pCMV-VSVG and pCMV-dR8.2) were cotransfected into HEK293T cells using Lipofectamine 2000 (Invitrogen; California). The conditioned media were collected at 48 and 72 h post-transfection, pooled, and filtered through a 0.45-μm filter. Filtered conditioned media were used to infect target cells in the presence of 8 μg/ml Polybrene. Cells were initially infected for 8 h, allowed to recover for 48 h in complete media, and then selected using puromycin over 24 h. The positive cells were continuously cultured for further analysis.

2.6. Antibody and immunoblotting

Cells were extracted using lysis buffer for Western blot and IP (Beyotime, China); the buffer was supplemented with 1% proteinase inhibitor cocktail (Sigma–Aldrich; Missouri), 25 mM NaF, and 1 mM Na_3VO_4. Membranes were incubated with the following primary antibodies: antiphosphotyrosine FAK (Tyr397, Tyr576/7, Tyr925), phosphorylated Src (pTyr419, (n-p)Tyr530), phosphorylated ERK (p-ERK), FAK, GAPDH, Src, and ERK, which were obtained from Cell Signaling Technology. Anti-HA monoclonal antibodies were obtained from Sigma.

2.7. Wound-healing and migration, invasion assay

In the wound-healing assay, cells that were infected with empty vector (NC), wild-type, or FAK-Del33-containing virus were used. Cells were initially seeded uniformly onto 60-mm culture plates with an artificial "wound" carefully created at 0 h using a P-20 pipette tip. Images of the wounds were recorded at 0 h and 24 h from three independent experiments. For Transwell assays, cells were serum-starved overnight in 0.1% bovine serum. Transwell filters (8 μm pore size; Corning) uncoated or coated with fibronectin (10 μg/ml) were used for migration or invasion assays. Cells (3×10^5) in serum-free medium containing 0.1% BSA were added to the upper chambers in triplicate. Then, 20% FBS DMEM was added to the bottom chamber. After 24 h incubation at 37 °C, non-migrated cells on the top of the filters were removed. Cells that had migrated to the bottom of the filters were fixed and stained using Giemsa.

2.8. Cell proliferation assay

Cell proliferation was determined using WST-8 dye (Beyotime, China) according to the manufacturer's instructions. Briefly, 5×10^3 cells/well were seeded in a 96-well flat-bottomed plate, grown at 37 °C for 24–96 h, and then 10 μl WST-8 dye was add to each well; cells were then incubated at 37 °C for 3 h, and the absorbance was determined at 450 nm using a microplate reader.

2.9. Apoptosis flow cytometry assay

The apoptotic cell population was determined using a flow cytometric assay. Briefly, 5×10^5 cells/well were seeded in a 6-well plate, grown at 37 °C overnight, and then placed in serum-starved conditions for a further 24 h. Subsequently, the cells were treated without or with apoptosis inducers A (Apopisa) and B (Apobid) (1:1000; Beyotime, China) in the presence of 10% FBS for 16 h. Cells were trypsinized, washed and re-suspended in PBS. Then, the cultures were stained with Annexin V-FITC and 7-ADD (BD Biosciences, USA), and apoptosis rates were analyzed using a flow cytometer.

2.10. Statistical analysis

Statistical analysis was performed using the SPSS software version 11.0 (Chicago, USA). Differences between the two groups were evaluated using a one-way analysis of variance. A chi-square test was used to calculate the significance of detection rates between two groups. Values of P that were less than 0.005 were considered significant.

3. Results

3.1. Novel FAK mutations in breast cancer

We used RT-PCR to amplify and sequence the coding region of oligo-dT primed FAK cDNAs from 25 paired tumor/normal tissues (6 breast cancer, 10 thyroid cancer, 5 colon cancer, 4 gastric cancers). Multiple independent cDNA plasmid clones were generated