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Antisense *Tiam1* Down-Regulates the Invasiveness of 95D Cells *in Vitro*

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Abstract As a specific guanine nucleotide exchange factor of Rac1, Tiam1 (T-lymphoma invasion and metastasis inducing protein 1) is involved in a number of cellular events, such as cytoskeleton reorganization, cell adhesion, and cell migration. Since Tiam1 was implicated in the invasion and metastasis of T-lymphoma cells and breast tumor cells, we compared the expression level of Tiam1 in two human giant-cell lung carcinoma cell strains with high or low metastasis potential, and found that Tiam1 expression level in high-metastatic 95D cells was higher than that in low-metastatic 95C cells. To further confirm the role of Tiam1 in invasion and metastasis, we constructed the antisense *Tiam1* expression plasmid (pcDNA3-anti-*Tiam1*), which was transfected into 95D cells. A stable transfected clone with decreased Tiam1 expression was screened and selected for further research. Transwell assay showed that down-regulation of endogenous Tiam1 by anti-*Tiam1* can reduce the *in vitro* invasiveness of 95D cells. Our results suggested that Tiam1 signaling contributed to the invasion and metastasis of the human giant-cell lung carcinoma cells.

Key words T-lymphoma invasion and metastasis inducing protein 1 (Tiam1); invasion and metastasis

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Invasion and metastasis are the main death causes of tumor patients, and aberrant expression of some genes contributes to tumor cell invasion and metastasis [1]. *Tiam1* was firstly identified as a gene amplified by inserted retrovirus which can confer metastatic capacity to the non-metastatic T-lymphoma cells [2]. Tiam1 contains a DH domain adjacent to PH domain, which is a typical structure of guanine nucleotide exchange factors (GEFs) [3]. As a Rac1-specific GEF, Tiam1 can catalyze the transition of Rac1 from inactive GDP-bound state to active GTP-bound state, and the active GTP-bound Rac1 is involved in many important cellular processes, such as cytoskeletal reorganization, cell adhesion and migration, gene expression, apoptosis and cell cycle [4].

In this study, we firstly compared the expression level of Tiam1 in the low-metastatic 95C and high-metastatic 95D cells. Furthermore, we investigated the effect of down-regulation of Tiam1 by stable transfection of pcDNA3-anti-*Tiam1* on the *in vitro* invasiveness of 95D cells. Our work will be helpful to clarify the role of Tiam1 in the invasion and metastasis of human giant-cell lung carcinoma cells.

Materials and Methods

Materials

Rabbit polyclonal antibody against human Tiam1 was purchased from Santa Cruz. Mouse monoclonal antibody against human α-tubulin was purchased from Newmarker. The HRP conjugated goat anti-rabbit or anti-mouse IgG were purchased from CNI. ECL was product of Pierce and X-ray films were from Kordak. RPMI 1640 medium, G418, T4 ligase and restriction endonucleases were purchased from Invitrogen.

Plasmid construction

Total RNA was extracted from 95D cells by Trizol reagent (Invitrogen) as recommended by the manufacturer. A 500 bp nucleotide sequence (–68 to +432) of Tiam1 was obtained by RT-PCR (Promega) from RNA of 95D cells and the fragment was inserted into pcDNA3 vector

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reversely using the indicated MCS. PCR primers (Sangon) were designed as the following: P1: 5′-CGG*GAATTC*TAA-ATGCCACAGTGC-3′ (*Eco*RI underlined); P2: 5′-CAA*GGATCC*CTCAGCCAAATATGTG-3′ (*Bam*HI underlined). DNA sequencing was performed by Genecore.

Cell culture and stable transfection

95C and 95D cells were cultured in RPMI 1640 supplemented with 10% NBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. 95D cells in 35 mm dishes were transfected with pcDNA3 or pcDNA3-anti-*Tiam1* by Lipofectamine. 72 h after transfection, the cells were passaged to 100 mm dishes and 800 mg/ml G418 was added. The screening period was two weeks until the negative control cells died off. Several clones were selected and cultured in new 60 mm dishes with 200 mg/ml G418 pressure for further research.

In vitro invasion assay

24 transwell units were used for monitoring *in vitro* cell invasion as described previously [5]. 8 μm porosity polycarbonate filters of the chambers were coated with 100 μl reconstituted basement membrane substance Matrigel (BD Pharmingen). 1×10^5 tumor cells were placed in the upper chamber of the transwell unit. RPMI 1640 containing 10% FBS was placed in the lower chamber of the transwell unit. After 24 h incubation at 37 °C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell invasion processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting method using H&E staining technique (cell numbers in six random areas were counted under light microscope, 100×). Each assay was set up in triplicate and repeated at least three times. All data were analyzed statistically by *t*-test and statistical significance was set at $P<0.05$.

Western blotting

Cells were lysed in Ripa buffer (1% NP-40, 0.1% SDS, 0.5% DOC, 150 mM NaCl, 10 mM Tris-HCl, and a protease inhibitor mixture) at 4 °C for 30 min. Equal volume of lysate were electrophoresed with SDS-PAGE (5%–8%). The separated proteins in gel were transferred to the NC membrane and the transfer quality was monitored by Ponceous S staining. Blocked by 5% low-fat milk/TBS for 1 h at room temperature, the membrane was incubated with the primary antibodies (1:500) for 1 h at room temperature. After 3 times washing with T-TBS, the membrane was incubated with the HRP-conjugated secondary antibody (1:500) for 1 h at room temperature. After 3 times washing, the membrane was developed with ECL and the specific bands were recorded by X-ray film.

Results

Comparison of the *in vitro* invasiveness of 95C and 95D cells

95C and 95D cells were subcloned from the PLA-801 human giant-cell lung carcinoma cell line, but they have different metastatic potentials [6]. We used a transwell assay to compare the *in vitro* invasiveness of these two cell strains. As shown in Fig. 1, 95D cells have higher *in vitro* invasiveness than 95C cells, which is consistent with the previous report and the results of tumor-cells transplanting experiments in nude mice [7]. Such two cell strains provide a good comparative model for the research of invasion and metastasis.

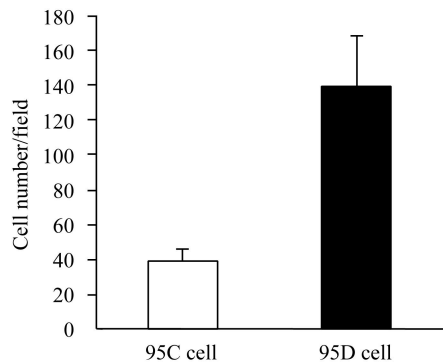

Fig. 1 95D cells have higher *in vitro* invasiveness than 95C cells

Comparison of Tiam1 expression level in 95C and 95D cells

Since Tiam1 was reported to be associated with tumor invasion and metastasis, we compared the expression level of Tiam1 in these two cell strains by Western blotting. The two cell strains both had high expression of Tiam1, but the level in 95D cells is higher than that in 95C cells (Fig. 2). Thus, our result indicated that Tiam1 expression level might be positively associated with the *in vitro* invasiveness of human giant-cell lung carcinoma cells.

Antisense *Tiam1* (anti-*Tiam1*) down-regulates Tiam1 expression level of 95D cells

To investigate the possibility that Tiam1 may regulate

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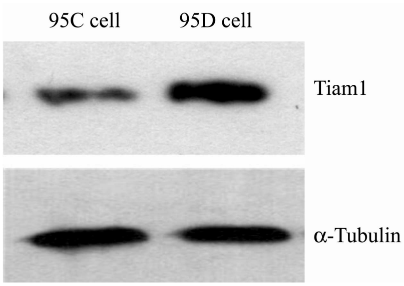

Fig. 2 Expression level of Tiam1 in 95D cells is higher than that in 95C cells

the *in vitro* invasiveness of 95C and 95D cells, we constructed the anti-*Tiam1* expression plasmid and transfected it into 95D cells, which has higher Tiam1 level and *in vitro* invasiveness. After G418 screening, Western blot analysis in the five selected clones showed that Tiam1 level in the No. 4 stable transfected clone was significantly decreased (Fig. 3).

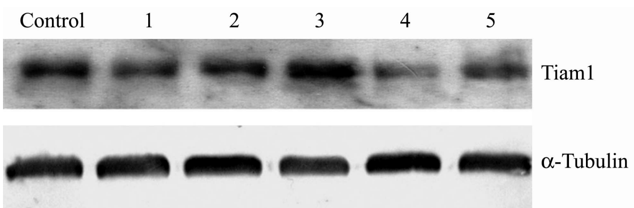

Fig. 3 Antisense *Tiam1* down-regulates Tiam1 expression level in 95D cells

Control, mock transfectants; 1–5, five clones selected from 95D cell stably transfected with pcDNA3-anti-*Tiam1*.

Antisense *Tiam1* down-regulates the *in vitro* invasiveness of 95D cells

We compared the *in vitro* invasiveness of the positive clone (No. 4) and mock transfectants. Transwell assay showed that antisense *Tiam1* could down-regulate the *in vitro* invasiveness of 95D cells (Fig. 4). Our result evidenced that Tiam1 contributes to the invasiveness of human giant-cell lung carcinoma cells.

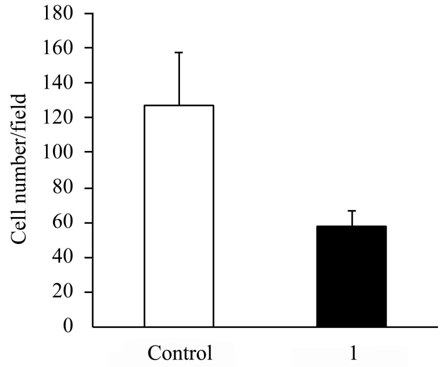

Fig. 4 Antisense *Tiam1* down-regulates the *in vitro* invasiveness of 95D cells

Control, mock transfectants; 1, 95D cells containing pcDNA3-anti-*Tiam1*.

Discussion

Tiam1 was first identified as an invasion- and metastasis-inducing factor in T-lymphoma cells and widely expressed in most tissues and tumor cell strains [2,8]. We found that in human giant-cell lung carcinoma cell strains 95C and 95D Tiam1 is easy to be detected by Western blotting. Although Tiam1 was found to bind to c-myc and down-regulate its transcriptional activity and serum-starvation induced apoptosis in Rat1 fibroblast, which is not Rac1-dependent [9], most function of Tiam1 is mediated by activation of Rac1 as one of its specific GEFs. Tiam1-Rac1 signaling can regulate cytoskeleton reorganization and gene transcription [10,11]. Engers *et al.* [12] reported that Tiam1 has no association with the *in vitro* invasion ability of human renal cancer cell lines and can down-regulate TMPIs expression in human renal cancer cells as well as Rac1-GTP. Tiam1 was also found to enhance the signaling from heregulin to β-catenin/LEF nucleotide transactivation and promote the invasion of human breast carcinoma cell strains; Tiam1 expression level is associated with the malignant grades of human breast carcinoma tissues [13].

Over-expression of Tiam1 C1199 in MDCK cells increased cell-cell adhesion, which contributed to decreased cell scattering ability [14]. Our research suggested that high expression of Tiam1 might partly promote invasion and metastasis of 95D cells. The function of Tiam1 on the *in vitro* invasion of 95D cells is consistent with the results of Bourguignon *et al.* [5,15], in which Tiam1 can interact with CD44 and ankyrin to promote the *in vitro* invasion of SP6 cells. One explanation of such contrary results is that carcinoma cells bearing high metastatic potential, in most cases, have already lost normal E-cadherin expression and localization. In the brain and testis tissues Tiam1 is also highly expressed but it doesn't lead to tumorigenesis [2], and the ambiguous reports of Tiam1 on tumorigenesis and metastasis remains largely characterized.

In conclusions, our results showed that down-regulation of endogenous Tiam1 by anti-*Tiam1* can reduce the *in vitro* invasiveness of 95D cells, which suggested that

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Tiam1 signaling played an important role in invasion and metastasis of human giant-cell lung carcinomas. The detailed molecular mechanism by which Tiam1 affects the invasion and metastasis in 95D cells needs further research.

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