RESEARCH ARTICLE

Effects of Tiam 1 on Invasive Capacity of Gastric Cancer Cells *in vitro* and Underlying Mechanisms

Jin-Ming Zhu[1]*, Pei-Wu Yu[2]

Abstract

Objective: To investigate changes in the invasive capacity of gastric cancer cells *in vitro* after expression inhibition of T lymphoma invasion and metastasis inducing factor 1 (Tiam 1) and underlying mechanisms. Methods: Using adhesion selection, two subpopulations with high (M_H) or low (M_L) invasive capacity were separated from the human gastric cancer cell line MKN-45 (M_0). Tiam 1 antisense oligodeoxynucleotide (ASODN) was transfected into M_H cells with liposomes, and expression of Tiam 1 mRNA and protein was determined by RT-PCR and quantitative cellular-ELISA. Changes in the cytoskeleton, invasive capacity *in vitro* and expression of ras-related C_3 botulinum toxin substrate 1 (Rac 1), integrin β1 and matrix metalloproteinase 2 (MMP 2) between Tiam 1 ASODN transfected M_H cells and non-transfected cells were observed by HE staining, cytoskeletal protein staining, scanning electron microscopy, Boyden chamber tests and cyto-immunohistochemistry. Results: A positive correlation existed between the expression level of Tiam l mRNA or protein and the invasion capacity of gastric cancer cells. After ASODN treatment (0.43 μM for 48 h), Tiam 1 mRNA transcription and protein expression in M_H cells were decreased by 80% and 24% respectively ($P < 0.05$), compared with untreated controls, while invasive capacity *in vitro* was suppressed by 60% ($P < 0.05$). Morphologic and ultrastructural observation also showed that ASODN-treated M_H cells exhibited smooth surfaces with obviously reduced filopodia and microspikes, which resembled M_0 and M_L cells. Additionally, cytoskeletal distribution dramatically altered from disorder to regularity with reduced long filament-like structure, projections, pseudopodia on cell surface, and with decreased acitn-bodies in cytoplasm. After Tiam 1 ASODN treatment, the expression of Rac 1 and Integrin β1 in M_H cells was not affected ($P > 0.05$), but that of MMP 2 in M_H cells was significantly inhibited compared with untreated cells ($P < 0.05$). Conclusion: Over-expression of Tiam-1 contributes to the invasive phenotype of gastric cancer cells. Inhibition of Tiam 1 expression could impair the invasive capacity of gastric cancer cells through modulating reconstruction of the cytoskeleton and regulating expression of MMP 2.

Keywords: Tiam 1 - gastric cancer - cytoskeleton - invasion

Introduction

Despite advances in surgical treatment, chemotherapy and radiotherapy, gastric cancer remains a major global health burden. The most recent estimates show that it is the fourth most common cancer and the second most common cause of cancer deaths worldwide (Lee et al., 2012; Mahar et al., 2012). Most victims of patients with gastric cancer died as a result of complications associated with recurrence and metastasis even after curative resection. But the invasion and metastasis of gastric cancer is a complex multistep process, in which invasiveness are necessary for gastric cancer cells to metastasize, and aberrant expression of some genes contributes to it (Lin et al., 2012).

T lymphoma invasion and metastasis inducing factor 1 (Tiam 1), an important member of Dbl (diffuse B-cell lymphoma) oncogene family, was firstly identified from T-lymphoma cells using proviral tagging in combination with in vitro selection for invasiveness (Habets et al., 1994). Huam Tiam 1 gene maps to the syntenic region (q22) on human chromosome 21 (21q22) and encodes a 170 kDa transmembrane glycoprotein with intrinsic Rho GTPases activity, which contains a DH (Dbl homologous) domain adjacent to PH (Pleckstrin homologous) domain, a typical structure of guanine nucleotide exchange factors (GEFs) (Habets et al., 1995). As a specific GEF for Rac 1 (ras-related C3 botulinum toxin substrate 1), a member of Rho oncogene family, Tiam l can catalyze the transition of Rac l from inactive GDP-bound state to active GTP-bound state, and then the latter one activates downstream signaling pathways related to many important cellular events, such as cytoskeletal reorganization, cell adhesion and migration (Mertens et al., 2003). Accordingly, Tiam 1 has been shown to increase invasion in T-lymphoma cells, as well as to stimulate cellular migration in fibroblasts,

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and to promote motility in some neurocytes. Increasing evidence has focused on Tiam 1's regulation, as well as Tiam 1's role in tumor progression and metastasis. Recent studies have demonstrated that increased Tiam 1 expression correlates with grade of breast cancer in humans and metastatic potential of human breast cancer cell lines in nude mice (Minard et al., 2004), so as in colon tumors, lung cancer, hepatocellular carcinomas and et al (Minard et al., 2006; Chen et al., 2012; Wang and Wang, 2012; Huang et al., 2013). Our clinical research showed negative staining of Tiam 1 protein in paracarcinoma gastric mucosa, obtained from patients with gastric cancer, whereas positive staining of Tiam 1 protein was detected in gastric cancer tissues (Zhu et al., 2005). Especially, as the degree of histologic differentiation of gastric cancer decreased, depth of invasion increased, stage of TNM upgraded or lymph node metastasis appeared, the staining of Tiam 1 protein in gastric cancer tissues became more intensive, which suggested Tiam 1 might be a candidate biomarker for judging the invasive and metastatic state of gastric cancer and forecasting the prognoses of patients, but the intrinsic biological mechanism is not clear.

Here, we tried to further define the mechanism of gastric cancer cell invasion induced by Tiam 1. Firstly, two subclones with high (M_H) or low (M_L) invasive and metastatic potentials, were isolated from human gastric cancer cell line MKN-45 (M_0) by adhesion selection in vitro, described as previous (Chen et al., 2001). We compared the expression level of Tiam l in them and the differences on their morphologic features, meanwhile correlations between the expression of Tiam 1 and the in vitro invasive potentials of these three cells were analysed. Then, we investigated the effect of Tiam 1 down-regulation by antisense oligodeoxynucleotides transfection on the changes in cytoskeletal structure of M_H cells, the invasiveness of M_H cells in vitro and the expression of Rac 1, Integrin β1 and MMP 2 in M_H cells. Our work will be helpful to provide a better insight into the mechanism of gastric cancer cell invasion and metastasis induced by Tiam 1, and the development of new treatment strategies in gastric cancer.

Materials and Methods

Cell Culture and Adhesion Selection

The parent cell line MKN-45 (M_0) is a poorly differentiated gastric adenocarcinoma cell line (Yokozaki, 2000), acquired from Shanghai Institute of Digestive Disease in Shanghai Second Medical University Renji Hospital, China. Frozen cell stocks were thawed and grown in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, NY), L-glutamine, sodium bicarbonate, essential amino acids, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and sodium pyruvate. The medium was replaced with fresh medium every 2-3 days to maintain optimal growth at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged at a ratio of 1:3 when they reached approximately 80% confluence, detached using a 0.25% (w/v) trypsin-EDTA solution every 5-6 days.

M_0 cells were selected for adhesion on mouse laminin 1 (Sigma-Aldrich, St Louis, MO, USA) coated substrates following the protocol previously described (Chen et al., 2001). Briefly, culture dishes (d = 35 mm) were coated with 50 μg laminin 1 in phosphate buffered saline (PBS) and air-dried overnight at 4°C. Subconfluent M_0 cells were incubated in 0.25% trypsin-EDTA solution, washed and resuspended in serum-free medium. The cell suspension (2.5×10^5/dish) was incubated with laminin 1 coated substrate for 1 h; attached and unattached cells were collected separately. The attached cells were cultured to subconfluence on laminin 1 coated dishes, then selected again for adhesion, so did with that unattached ones. After adhesion selections were performed serially 20 times, the attached cells (M_H) or unattached cells (M_L) were then maintained under normal culture conditions. Selected cells (M_H and M_L) were used for experiments after at least 2 passages following the last adhesion selection.

Antisense Treatment of Highly Invasive Subclone (M_H)

Design of one 18-mer antisense oligodeoxynucleotide (ASODN) was based on the nucleotide sequence of human Tiam 1, targeted to Dbl homology (DH) domain, while sense oligonucleotide (SODN) used as a control. The primers of ASODN and SODN derived from published sequences previous (Li et al., 2000), were specific, determined using a BLAST search of current EMBL database. The sequences were: SODN 5'-*G*AT CTG CGA GCT CCT GG*A*-3'(* represents phosphorothioate linkages); ASODN 5'-*T*CC AGG AGC TCG CAG AT*C*-3'. All of ODNs were synthesized using an Applied Biosystems 391 DNA synthesiser (Perkin Elmer Applied Biosystems, Inc., Foster City, USA).

M_H cells in log phase growth were plated onto culture dishes (d = 35 mm) at quantities of 2.5×10^5 cells and incubated at standard culture conditions for 24 h, then transfected with ASODN or SODN of Tiam 1 by DOTAP liposomal transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany). The preparation of liposome-ODN incorporation was carried out according to manufacture's protocol. Before treatment, ODNs and Liposomes (ratio w/v = 5 μg/ 15 μl = 1:3) were dissolved separately in HBS buffer (Hepes buffered saline: 20 mM HEPES, 150 mM NaCl, pH 7.4), then mixed together at room temperature for 15 min. Optimal working condition of Lipofectin-ODN was determined previously in preliminary experiments (datas not shown). Experimental cells were treated with liposome-ASODN at a final concentration of 0.43 μM. Control treatments included serum-free culture medium only, Liposome only (15 μl/dish) and Liposome-SODN (0.43 μM). The culture medium in dishes of cells was removed and the cells exposed to lipofection solution for 8 h. At the end of the incubation with ODN-liposome, the medium was replaced with fresh serum-containing culture medium (3 ml/dish) before placement into incubator. Tiam 1 mRNA expression assay of M_H cells was used after transfection for 48 h.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted according to the instruction

of Tripure Isolation Reagent (Boehringer Mannheim, Germany), the content and purity of which were evaluated with ultra-violet spectrophotography. cDNA was synthesized in 20 μl reaction volumes containing 1μg of total RNA, 0.2 μg/μl random hexamer primer, 4 μl 5 × reaction buffer, 20 U/μl ribonuclease inhibitor, 10 mmol/L deoxyribonucleotide triphosphate (dNTPs) and 20 U Moloney murine leukemia virus (M-MuLV) reverse transcriptase. All of the procedures were made according to the instruction of First Strand cDNA Synthesis Kit (Sangon Biology Engineering Co., Shanghai, China).

For semi-quantitative assay, a standard calibration curve was set up to determine optimum number of cycles (15-35 cycles). 30 cycles revealed to be most appropriate (Datas not shown). PCR was carried out with the following materials: 2 μl cDNA, 5 μl 10 × buffer, 4 μl 25 mmol/L MgCl2, 8 μl 2.5 mmol/L dNTPs, 0.5 μl 25 μmol/L of each forward and reverse amplification primers, and 0.5 μl (2.5 U) Takara Ex Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China) in a 50 μl reaction volume. Reactions were carried out in PTC-100TM PCR system (MJ Research Inc., Watertown, MA, USA). PCR conditions were, therefore: 30 cycles; 94°C (45 s), 54°C (1 min), 72°C (1.5 min). The following primer pairs (synthesized by Sangon Biology Engineering Co., Shanghai, China.) were used to amplify fragments of Tiam 1 (818 bp) and GADPH (glyceraldehyde-3-phosphate dehydrogenase, 310 bp) cDNAs: Tiam 1: forward primer 5'-TTC TCA CCA GTC TGT TCA GC-3'; reverse primer 5'-CCA GAC TTG GAA TCC TCA GA-3'; GADPH: forward primer 5'-AGG TCC ACC ACT GAC ACG TT-3'; reverse primer 5'-GCC TCA AGA TCA TCA GCA AT-3'.

5 μl PCR products amplified from Tiam 1 or GADPH gene respectively were mixed and then underwent a 2% ethidium bromide stained agarose gel electrophoresis along with molecular weight standards. The band intensities for Tiam 1 and GADPH were estimated with a Bio-Rad gel documentation 2000 system (Bio-Rad Co., USA). The intensities of Tiam 1 gene expression were reported as the ratio (RV) of Tiam 1 to GADPH (as an internal control) on the expression quantities. Specimens were analyzed three times and the values were averaged.

Quantitative Cellular-ELISA

M_H cells in log phase growth were plated onto 96-well plates at quantities of 1×10^5 cells and incubated at standard culture conditions for 24 h, then transfected as stated above, so did the control groups. Quantitative cellular-ELISA of Tiam 1 protein in gastric cancer cells was used after transfection for 48 h, referring to the procedure reported in previous literature (Zhang et al., 2001).

In brief, the culture medium in 96-well plates was removed, washed twice with PBS, fixed with 4% paraformaldehyde (w/v in PBS) for 1 hour and removed. After washed three times with PBS, cells were blocked with 10% (w/v) goat serum albumin in PBS at 4°C for 15 min, and then incubated at 37°C for 2 h with a 1:100 working dilution of rabbit polyclonal antibody (50 μL/well) against human Tiam 1 (Santa Cruz, USA). Followed by three washes with 0.05% Tween-20 / PBS,

the cells were incubated at 37°C for 1 h with a 1:5000 (50 μL/well) working dilution of Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Zhongshan Biotechnology CO., Ltd., Beijing, China). After four washes with 0.05% Tween-20/PBS, 3, 3', 5, 5'-tetramethylbenzidine (TMB) peroxidase chromogenic reagent (KPL, Gaithersburg, Md. America) of A and B fluid with equivalent amount blending was added (100 μL/well) in dark for 10 min, then the reaction was stopped with 1 mol/L HCl 50 μl/well with oscillation for 30 s and the absorbance was measured using an ELISA plate reader (Sunrise, Tecan Group, Ltd., Switzerland) at 450 nm. Followed by distilled water washing, 0.08% (w/v) crystal violet (100 μL/well) incubated at room temperature for 20 min, distilled water washing again, the reaction was stopped with 33% (w/v) glacial acetic acid (100 μL/well) at room temperature for 30 min and the absorbance was measured at 550 nm. The intensities of Tiam 1 protein were reported as the ratio (RD) of D_{450} to D_{550}. Specimens were analyzed three times and the values were averaged. Negative controls consisted of omission of the primary antibody.

Morphologic and Ultrastructural Observations

To examine morphological changes, cells directly cultured on slides were washed twice with PBS, stained with hematoxylin and eosin (H&E), then viewed under light microscope and representative pictures were taken. For scanning electron microscopy (SEM), cells grown on cover slips were fixed with 2.5% (w/v) glutaraldehyde at 4°C overnight. After washed in PBS, the cells were treated with 1% (w/v) osmium tetroxide (TAAB Laboratories Equipment Ltd. Berkshire, UK) at 4°C for 1 h, washed three times with PBS, and dehydrated with a gradient ethano1. SEM specimens were dried using a critical point dryer (Hitachi Hcp-2), and the sputter was coated with Pt+Cd using an ion sputering device (Hitachi Oie-102). The cell surface alteration was observed under a SEM device (Amray 1000B).

Cytoskeletal Staining

Cytoskeletal staining was conducted as described previous (Zhang et al., 1996). After washed twice with PBS, Cells cultured on slides were permeabilized with 0.1% Triton-X-100 for 3×8 min, washed in M-buffered solution (containing imidazole 50 mM, potassium chloride 50 mM, magnesium chloride 0.5 mM, EGTA 2mM, EDTA 0.1 mM, mercaptoethanol 1 mM, glycerol 4M, and distilled water metered volume to 1,000 ml) for 3×4 min, and then fixed with 3% (w/v) glutaraldehyde at room temperature for 10 min. After washed with M-buffered solution for 3×3 min, cells were stained with a Coomasssie Brilliant Blue solution (containing R250 0.2 g, methanol 46.5 ml, glacial acetic acid 7 ml, and distilled water 46.5 ml) at room temperature for 40 min, followed by PBS washing, air drying, dimethyl benzene clearing, neutral gum sealing, and then viewed under light microscope (Olympus optical Co., Model LH50A, Japan).

In Vitro Invasion Assay

24-transwell units (Milipore, USA) were used for

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monitoring in vitro cell invasion as described previous (Albini et al., 1987), 12 μm diameter polyvinylpyrrolidone-free polycarbonate filters with 8 μm pore size (Milipore, USA) in which, were coated with 30 μg of Matrigel (BD Biosciences, Canada) per filter. NIH-3T3 cell conditioned media as a chemoattractant, was obtained by culturing cells for 24 h in serum-free RPMI 1640. Cells in serum-free RPMI1640 medium (400 μl containing 1×10^5 cells) were added to the upper compartment of the chamber, while 600 μl of conditioned medium was added to the lower compartment. After 72h incubation at 37°C in a humidified 95% air with 5% CO_2, basement membrane Matrigel and cells on the upper side of the filter were removed by wiping with a cotton swap, and cells migrating to the lower surface of the filters were fixed and stained with H&E. The invasive potential of cells was determined by measuring cells that migrate to the lower side of the filters through the basement membrane matrigel and pore. The number of cells that penetrated the filter was counted in 10 microscopic fields of each filter under 200× magnification. Assays were carried out in triplicates.

Cyto-Immunohistochemistry

M_H cells in log phase growth were plated onto 6-well plates preset with slides at quantities of 2×10^6 cells and incubated at standard culture conditions for 24 h, then transfected as stated above, so did the control groups. Cyto-immunohistochemistry of Rac 1, Integrin β1 and MMP 2 protein in gastric cancer cells was used after transfection for 48 h.

In brief, the culture medium in 6-well plates was removed, washed twice with PBS, fixed with acetone for 10 min and removed. After washed two times with PBS, incubated with 3% H_2O_2 for 10 min and washed with PBS, cells were blocked with 10% (w/v) goat serum albumin in PBS at 4°C for 15 min, and then incubated at 37°C for 1 h with a 1:100 working dilution of rabbit polyclonal antibody (100 μL/well) against human Rac 1 (Santa Cruz, USA), so did the rabbit polyclonal antibody against human Integrin β1 and mouse monoclonal antibody against human MMP 2 (Santa Cruz, USA). Cells were washed in PBS and processed using the SABC (StreptAvidin Biotin Complex) amplified system and the DAB (Diaminobenzidine, colouring as yellow or blue) or AEC (3-Amino-9-Ethylcarbazole, colouring as red) colour-developing agent (Boster Biotechnology CO., Ltd., Wuhan, China) according to the manufacturer's suggested procedure. Then the cell climbing films were dried and mounted for Microscopy. A fine granular pattern staining of the antigens was observed in both cytomembrane and cytoplasm. Using MIG2000 Image Analysis Measured System, 100 cells were randomly selected and the total area of positive staining cells and its integral optical density (IOD) was calculated separately. The intensities of Rac 1, Integrin β1 and MMP 2 protein were reported as the ratio (D_λ) of IOD to Area. Specimens were analyzed three times and the values were averaged. PBS instead of primary antibody as negative control.

Statistical Analysis

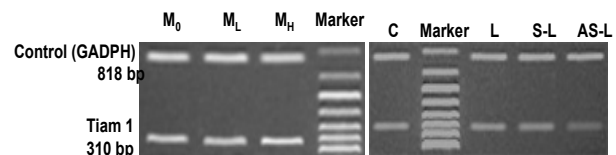Quantitative datas were presented as Mean ± SD. All

Figure 1. Expression of Tiam 1 in Gastric Cancer Cells by RT-PCR Analysis. A: M_H cell subclone expressed Tiam-1 strongly, while M_0 and M_L cells expressed faint bands. B: ASODN-liposome treated M_H cells (AS-L) expressed Tiam-1 faintly, while untreated (C), only liposome treated (L) and SODN-liposome treated (S-L) M_H cells expressed strong bands

datas were analyzed statistically by One-way ANOVA followed by LSD (least significant difference) test, using SPSS10.0 software program (SPSS Inc., Chicago, IL), and difference was considered significant when $P < 0.05$. Correlations were calculated using the Spearman correlation coefficient.

Results

Comparison of the Invasive Potentials among M_0, M_L and M_H Cells In Vitro

M_H and M_L cells were subcloned from human gastric cancer MKN-45 cell line (M_0) by adhesion selection, which had different invasive and metastatic potentials. We used a transwell assay to compare the invasiveness of these three cell strains in vitro. M_H cells (24.33 ± 8.02) had higher invasive potential than M_0 (11.67 ± 3.79) and M_L (9.67 ± 3.06) cells in vitro (P < 0.05), which was consistent with the previous report and the results of tumor-cells transplanting and experimental metastasis in nude mice (Chen et al., 2001).

Comparison of the Levels of Tiam-1 mRNA expression and Protein among M_0, M_L and M_H Cells

Since Tiam l was reported to be associated with tumor invasion and metastasis, we compared the expression level of Tiam l mRNA and protein in M_0, M_L and M_H cells by RT-PCR (Figure 1) and Quantitative Cellular-ELISA. The expression of Tiam 1 mRNA and protein in these three strains were positive, but the level in M_H cells (RV = 0.855 ± 0.051, RD = 1.262 ± 0.165) is much higher than that in M_0 cells (RV = 0.759 ± 0.047, RD = 0.911 ± 0.104) and M_L cells (RV = 0.743 ± 0.039, RD = 0.892 ± 0.101) (P < 0.05).

After further investigation on the above datas, there was a positive correlation existed between the expression levels of Tiam l mRNA or protein and the invasion capacities of these cells (Spearman correlation coefficient r = 1.000, P < 0.05).

Antisense Treatment Down-regulated the Expression of Tiam 1 mRNA and Protein in M_H Cells

In order to confirm the effect of Tiam 1 ASODN, we carried out RT-PCR and Quantitative Cellular-ELISA in M_H cells. The optimum conditions for antisense treatment, determined in preliminary experiments (data not shown), were 0.43 μM ODN, ratio (w/v) of ODN/liposome = 1:3, 4 h of exposure, 48 h of recovery. After ASODN-liposome treatment, expression of Tiam 1 in M_H cells was significantly inhibited compared with those untreated
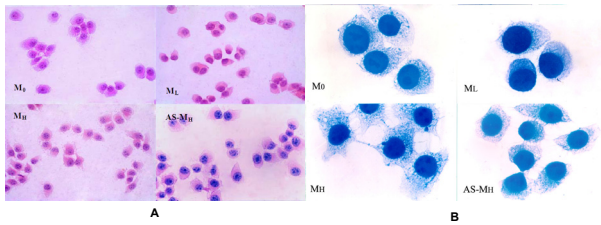
Figure 2. Cellular Morphology and Cytoskeletal Structure of Gastric Cancer Cells. A: H&E stain of M_0, M_L, M_H and antisense treatment M_H (AS-M_H) cells were observed by light microscopy. Magnification 200×. B: Cytoskeletal stain of of M_0, M_L, M_H and AS-M_H cells were observed by oil lens microscopy. Magnification 1000×
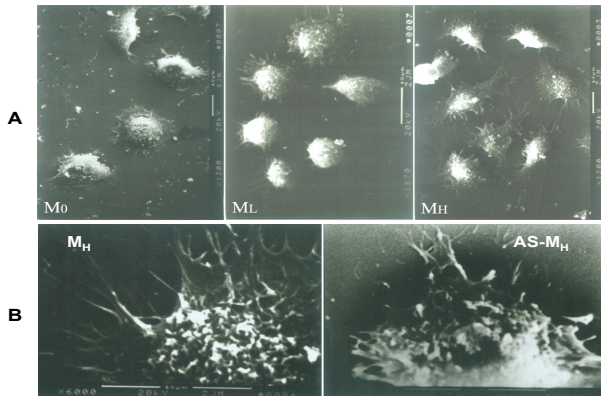

Figure 3. Ultrastructure of Gastric Cancer Cells Viewed by SEM. A: Ultrastructural features of gastric cancer cells. B: Ultrastructural alterations of M_H cells after antisense treatment

Figure 4. Comparison of the expression level of Rac 1, Integrin β1 and MMP 2 protein among M_L cell, Tiam 1 ASODN transfected M_H cell and no transfected M_H Cell. Compared with M_L group, * $P < 0.05$; Compared with transfected M_H group, $\triangle P < 0.05$

Figure 5. Cyto-Immunohistochemical Detection of Rac 1, Integrin β1 and MMP 2 protein in gastric cancer cells (Magnification 200×). A: Rac 1, B: Integrin β1, C: MMP 2. 1: M_L cells, 2: transfected M_H cells, 3: M_H cells

(Control), only liposome treated or SODN-liposome treated ones. RT-PCR showed that only faint Tiam 1 band was observed in M_H cells after ASODN treatment (RV = 0.162±0.018), whereas it was much stronger in the untreated (RV = 0.801±0.065), only liposome treated (RV = 0.789±0.054) or SODN-liposome treated groups (RV = 0.754±0.039) ($P < 0.05$) (Figure 1). Quantitative Cellular-ELISA of M_H cells transfected with ASODN-liposome (RD = 0.982 ± 0.119) also exhibited a significant reduction in Tiam 1 protein level, relative to untreated (RD = 1.290 ± 0.182), only liposome treated (RD = 1.237 ± 0.108) or SODN-liposome treated groups (RD = 1.234 ± 0.103) ($P < 0.05$).

Features of Cellular Morphology, Cytoskeletal Structure and Ultrastructure and the Alterations after Antisense Treatment

After stained with H&E and viewed under light microscope (200×), M_0 and M_L cells were floating and round epithelial-like morphology with large nucleo-cytoplasmic ratio, no obvious differences found between them. But multiform in M_H cells vs uniform in M_0 and M_L cells, some of M_H cells showed polygonals and had much longer cytoplasmic processes (Figure 2). After Tiam-1 ASODN treatment, the morphology in M_H cells resembled that in M_0 and M_L cells.

Scanning electron microscopy (SEM) revealed abundant microvilli, cytoplasmic processes and big microhills dotted on the surface of M_H cells, and some of the processes extended far, radiated in all directions or formed bulges at the end. But those on M_0 and M_L cells
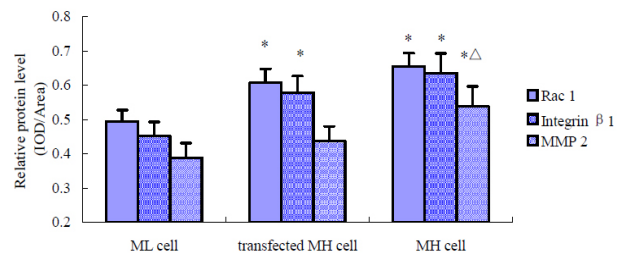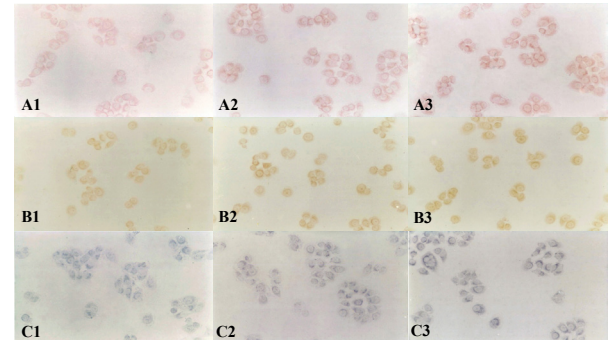
were short and compact. No obvious desmosomes, tight conjunction or other cell junction structures were observed in these three cells. After antisense treatment, M_H cells exhibited smooth surface with obviously reduced filopodia and microspikes (Figure 3).

Stained with coomasssie brilliant blue R250 and viewed under immersion objective, cytoskeletal structure of M_0, M_L and M_H cells formed a complicated cytoskeletal network in cytoplasm, particularly more concentrated in perinucleus areas, and bundles of cytoskeletal filaments stretched and scattered intercellularly. Compared with M_0 and M_L cells, in a whole, the cytoskeletal alignment in M_H cells exhibited somewhat disorder, accompanied with more dotted actin-bodies, cytoplasmic processes and long filament-like structures, whereas no obvious differences observed between the fore two cells. Afer antisense treatment, cytoskeletal distribution in M_H cells dramatically changed from irregular to regular with reduced long filament-like structure on cell surface and dotted acitn-bodies in cytoplasm, which resembled that of M_0 and M_L cells (Figure 2).

Tiam 1 ASODN Treatment Down-regulated the Invasiveness of M_H cells In Vitro

In vitro transwel1 assay showed the number of antisense-treated M_H cells, penetrating the artificial basement membrane, were 10.33 ± 2.52 cells per high power field (Magnification 200×), which was significantly lower than that of untreated M_H cells, 25.67 ± 6.11 per high power field ($P < 0.05$).

Effect of Tiam 1 ASODN Treatment on the expression of Rac 1, Integrin β1 and MMP 2 in M_H Cells

In order to explore the underlying cellular mechanism of Tiam 1 on the invasive capacity of gastric cancer cells, Cyto-Immunohistochemistry was performed. After Tiam 1 ASODN treatment, the expression of Rac 1 in M_H cells was not significantly inhibited compared with those untreated cells, but both transfected and no transfected M_H cells had a significantly higher level of Rac 1 protein than M_L cells. So did the expression of Integrin β1. Whereas, after Tiam 1 ASODN treatment, the expression of MMP 2 in M_H cells was significantly inhibited compared with those untreated cells, meanwhile, only no transfected M_H cells had a significantly higher level of MMP 2 protein than M_L cells (Figure 4~5).

Discussion

Tumor cell invasion and metastasis depends on a set of unique biological properties that enable malignant cells to complete all the steps in metastatic cascade. A tumor mass commonly consists of different cell subclones, a phenomenon known as tumor heterogeneity (Lleonart et al., 2000). Since subclones are from the same parent cell line, thus having a same genetic background, the differences in their phenotypes must have some underlying molecular mechanisms. In-depth study on their differences might help us gain new insight into the mechanisms of tumor cell invasion and metastasis.

It is well kown that the Rho family of GTPases have emerged as key players in regulating a diverse set of biological activities including actin organization, focal complex/adhesion assembly, cell motility, cell polarity, gene transcription and et al, the importance of which in cancer progression, particularly in the area of metastasis, is becoming increasingly evident (Etienne and Hall, 2002; Malliri and Collard, 2003). As an upstream regulator of Rho-GTPases, especially in the activity of Rac 1, the possible role of Tiam 1 in tumor invasion and metastasis also becomes more and more outstanding.

So in this study, we firstly acquired two subclones with high (M_H) or low (M_L) invasive and metastatic potentials from human gastric cancer cell line MKN-45 (M_0) using in vitro adhesion selection on laminin 1 coated dishes (Chen et al., 2001), then compared the expression level of Tiam 1 in them. Our study had shown the expression level of Tiam 1 mRNA and protein in M_H cells was much more intensive than that in M_0 and M_L cells, simultaneously a positive correlation existed between the expression of Tiam 1 and the in vitro invasive potentials of M_0, M_L and M_H cells. This result was consistent with that we had observed in clinical studies (Zhu et al., 2005), and all the above strongly implicated a role for Tiam 1 in the invasive process of human gastric cancer.

Additionally, the cytoskeletal ingredients of microtubules, microfilaments and intermediate filaments, et al., are responsible for the maintainance of cell morphology, direction of cell locomotion, cell spreading, cell migration and stability of cell-substrate contact in normal cells (Hirohashi and Kanai, 2003). Alterations in cytoskeletal structures including reduced microtubules, disruption of stress fibers and redistribution of actin-filaments in tumor cells have been documented, which

play in relation to the phenotype of tumor cell malignancy and benefit for them to transform and transmigration in tissue space (Ben-Ze'ev, 1985). Therefore, it is intriguing to investigate the cytoskeletal changes in tumor cells with special respect to their invasive and metastatic capabilities. Our observations in the cellular morphology and ultrastructure of gastric cancer cells had shown that M_0 and M_L cells were floating and round epithelial-like, but multiform in M_H cells, some of which showed polygonals and had much longer cytoplasmic processes. SEM also revealed abundant microvilli, cytoplasmic processes and big microhills dotted on the surface of M_H cells, and some of these processes extended far, radiated in all directions or formed bulges at the end, compared with those short and compact ones on M_0 and M_L cells. After stained with coomasssie brilliant blue, the cytoskeletal structure in M_0, M_L and M_H cells was viewed to form a complicated cytoskeletal network in cytoplasm, particularly more concentrated in perinucleus areas, and bundles of cytoskeletal filaments passed through cell wall and connected together with cytoskeletal arrays of adjacent cells. Compared with M_0 and M_L cells, the cytoskeletal alignment in M_H cells exhibited somewhat disorder, accompanied with more dotted actin-bodies, cytoplasmic processes and long filament-like structures, whereas no obvious differences observed between M_0 and M_L cells. It was noticeable that the differences in cytoskeletal structure might be involved in cytodynamic activities related to dissemination and implantation which were based on the invasive and metastatic potentials of M_0, M_L and M_H cells. Considering Tiam 1 can regulate the reorganization of cytoskeletal structure, the differences in morphologic features of gastric cancer cells with different invasive and metastatic potentials might be related to the expression level of Tiam 1 in them.

Antisense oligodeoxynucleotides (ASODNs), binding and inactivating specific RNA sequences, is one of the best tools for studying gene function, regulation of gene expression, and interactions between gene products. Intracellular delivery of ASODNs results in the down-regulation of target gene expression by inhibiting transcription or translation without affecting other cellular functions. In order to validate the role of Tiam 1 in gastric cancer cell invasion, we attempted to apply a 18-mer ASODN to block its expression in M_H cells, transfected by DOTAP liposomal reagent, then observed the sequent changes in cellular morphology and in vitro invasiveness of M_H cells. As unmodified phosphodiester oligomers are susceptible to nucleases, a partially phosphorothioate oligomers were used, which relatively nuclease-resistant. Our studies demonstrated treatment of M_H cells with ASODNs (0.43 μM, 48h) decreased Tiam 1 mRNA transcription and protein expression by 70% [(0.801-0.162)/0.801] and 65% [(1.290-0.982)/1.290] respectively, compared with untreated controls. Meantime, the in vitro invasive potential of ASODN-treated M_H cells was suppressed by 75% [(25.67-10.33)/25.67], but SODN or lipofectin alone treatment was inactive. Morphologic and ultrastructural observations also exhibited that ASODN-treated M_H cells exhibited smooth surface with obviously reduced filopodia and microspikes, which

resembled M_0 and M_L cells. The cytoskeletal distribution in ASODN-treated M_H cells dramatically altered from disorder to regularity with reduced long filament-like structure, projections, pseudopodia on cell surface, and with reduced acitn-bodies in cytoplasm. Additionally, using Cyto-Immunohistochemistry, we observed Tiam 1 ASODN treatment had no effect on the expression of Rac 1, in spite of a significantly higher level of Rac 1 protein in both transfected and no transfected M_H cells than that in M_L cells. These results inversely verificated the previous hypothesis that Tiam 1 could induce gastric cancer cells invasion through regulating cytoskeletal reorganization, which based on the role of Tiam 1 in inducing the activity of Rac 1, instead of affecting the expression of Rac 1. The depolymerization of microtubules and microfilaments ultimately results in the disordered, actively motile properties, the detaching tendency and the metastatic potential of gastric cancer cells.

Furthermore, abnormalities in the expression and functional activity of cell adhesion molecules are implicated in the invasive and metastatic progression of tumor cells. Researches have proven that the intercellular and cell-matrix adhesion molecules are more than just cementing substances but regulate cell polarity, differentiation, invasion and migration (Okegawa et al., 2004). Many of these cellular events are mediated through their intimate association with cytoskeletal network, and the interactions between cytoskeletal network and the manipulation of its related molecules should be unraveled (Marhaba and Zoller, 2004). So the cell-model of laminin 1 adhesion selection in vitro, used in our experiment, may be a simple and time-saving method to screen a particular phenotypic cell subpopulation and would be suitable for the further understanding of the above quesitons.

As we all know, laminin 1, a major basement membrane glycoprotein, has been shown to promote the malignant phenotype and the expression of certain laminin receptors (LNR) has been correlated with the malignant character of tumor cells (Ekblom et al., 2003). Adhesion of cells to laminin 1 induces collagenase IV production, which is necessary for tumor cells to metastasize. In our research, we observed the expression of MMP 2 (type IV collagenase/gelatinase A) in M_H cells was significantly higher than that in M_L cells, but the level of MMP 2 was remarkably inhibited in M_H cells after Tiam 1 ASODN treatment. Earlier archives had demonstrated that Rac 1 was a mediator of MMP 2 activation in three-dimensional collagen gel and that MMP 2 activation was required for Rac 1-promoted cell invasion through collagen barrier (Zhuge and Xu, 2001). As an upstream regulator of Rac 1, besides of activating Rac 1, whether Tiam 1 through other pathways regulate the expression of MMP 2, it is also a mystery and further study should be done.

Our study also had shown that adherent subclone (M_H), isolated from gastric cancer cell line MKN-45 by in vitro adhesion selection on laminin 1, was more invasive and metastatic in vitro and in vivo than either parental cells (M_0) or non-adherent subclone (M_L), which suggested that the properties of these cells are closely related to their adhesiveness to the basement membrane and extracellular matrix (Chen et al., 2001). Previous findings suggested

that the increased invasive and metastatic potentials of laminin adhesion-selected subpopulation might be due to an alteration in the membrane distribution and/or affinities of multiple laminin receptors including 67 KDa laminin receptor and Integrin β1 (Kim et al., 1994-1995; Kim et al., 1998). Some other researchers indicated that Tiam 1 was a key molecule in Integrin-mediated activation of Rac, which is essential for proper production and secretion of laminin, a requirement for cell spreading and migration (Hamelers et al 2005; Cruz-Monserrate et al., 2008). In this research, we did not observed any significant effect of Tiam 1 on the expression of Integrin β1, which from the other side supported previous research achievements. Moreover, recent archives had shown that recruiting Tiam 1 to Rac 1-activating Integrin complexes played an important role in cell spreading and migration (O'Toole et al, 2011).

In summary, considering that the cancer cell population, either as a solid tumor mass in vivo or as a continuous cell line in vitro, is an ever-changing entity due to their genetic instability and selective environmental pressure, and the fact that Tiam 1 expression is related to the invasive potential of gasitri cancer cells, we presumed there might be some crosstalks among the expression of Tiam 1, the rearrangment of cytoskeletal structure and the expression and/or redistribution of cell surface adhesion molecules, meanwhile the related mechanisms of cellular signal transduction needed to be fully elucidated too.

Acknowledgements

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