Overexpression of Tiam1 promotes the progression of laryngeal squamous cell carcinoma

SHUANG WANG*, SHISHENG LI*, QINGLAI TANG, SHU YANG, SHUHUI WANG, JIAJIA LIU, MI YANG and XINMING YANG

Department of Otolaryngology, Head and Neck Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, P.R. China

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Abstract. T-lymphoma invasion and metastasis-inducing factor 1 (Tiam1) has been reported in various types of human cancer, which play important roles in facilitating the metastasis of malignant tumor. However, the investigation of Tiam1 in laryngeal squamous-cell carcinoma is extremely rare. The aim of the present study was to assess Tiam1 expression and examine its function in tumorigenesis and the metastasis of laryngeal squamous cell carcinoma (LSCC) *in vitro*. Tiam1 expression in 98 primary LSCC tissue specimens was analyzed by immunohistochemistry and correlated with clinicopathological parameters and patients' survival. To investigate the effects of Tiam1 on the progression of LSCC, Tiam1/C1199 plasmid was transfected into LSCC, and proliferation, apoptosis, migration and invasion of transfected cells were examined using MTT, flow cytometry, wound-healing and Transwell assay, respectively. The results showed that, Tiam1 was detected in all primary LSCC samples. Additionally, Tiam1 overexpression was closely correlated with tumor progression and patient survival. Tiam1 overexpression was statistically significant, and served as an independent predictor of prognosis for patients with LSCC. The upregulation of Tiam1 by Tiam1/C1199 plasmid had no effect on the proliferation of transfected cells, but decreased the apoptotic rate of transfected cells, while the ability of migration and invasion was increased. These results suggested that Tiam1 overexpression in LSCC is possibly involved in the promotion of migration and invasion, and is a promising therapeutic target in the prevention of the progression of LSCC.

Correspondence to: Dr Xinming Yang, Department of Otolaryngology, Head and Neck Surgery, The Second Xiangya Hospital, Central South University, 139 Renmin Road, Changsha, Hunan 410011, P.R. China
E-mail: x16y2013@outlook.com

*Contributed equally

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common tumor occurring in the head and neck, and accounts for ~5% of all cancer cases (1). Despite advances in the development of conventional therapies such as surgery, radiotherapy and chemotherapy, the 5-year survival rate for patients with LSCC remains unsatisfactory (~50-70%) (2). Locoregional recurrences, lymph node and distant metastasis are major causes of death that significantly affect prognosis in LSCC patients (3). Therefore, the recognition and identification of tumor markers associated with recurrence and/or metastasis is of great value in the prediction of malignant biological behavior and the direction of therapeutic strategies.

T-lymphoma invasion and metastasis-inducing factor 1 (Tiam1), as an invasion and metastasis-inducing gene, was first identified by proviral tagging in combination with *in vitro* selection for invasiveness from a murine T-lymphoma cell line (4). The Rho-like small guanosine triphosphate (GTP)-binding proteins, as molecular switches, cycle between an inactive and an active GTP-bound state. Guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, positively stimulte these GTP-binding proteins in response to various signaling pathways (5). Tiam1, as a member of Dbl family proteins, is a specific GEF for the Rho-like GTP-binding protein Rac, which is involved in cell migration, invasion and metastasis (6).

Tiam1 overexpression is prevalent in numerous solid tumors, including those of prostate (7), hepatocellular (8,9), nasopharyngeal (10), esophageal (11) and renal cell carcinoma (12), colorectal cancer (13) and gallbladder carcinoma (14). Tiam1 is also reported to have intimate correlations with apoptosis (15), invasion and migration (16,17). Therefore, the overexpression of Tiam1 is important in the progression of malignant tumors. However, to the best of our knowledge, few studies evaluating the role of Tiam1 protein expression in LSCC are available. Consequently, we investigated Tiam1 expression in a number of LSCC tissue samples and assessed whether Tiam1 expression was correlated with clinicopathological parameters and prognosis in LSCC patients. Moreover, Tiam1/C1199 plasmid was employed to upregulate Tiam1 expression in LSCC cell lines, and the roles of Tiam1 in LSCC cell growth, apoptosis, migration, and invasion *in vitro* were investigated.

Materials and methods

Tissue samples and patients. A total of 98 patients with LSCC identified in the Department of Otolaryngology Head and Neck Surgery of Second Xiangya Hospital in Central South University from January 2003 to December 2006 were studied retrospectively. All 98 included patients had no history of radiotherapy or chemotherapy. All patients received treatment with curative intent and definitive resection and/or therapeutic comprehensive neck dissection, and/or post-surgical radiotherapy or chemotherapy. After the completion of treatment, the patients underwent routine surveillance every 1-3 months. Four patients were lost to follow up due to home moving or telephone number changes. Recurrence and metastasis were determined by physical examination, imaging evaluation, operation and postoperative pathological examinations. Overall and disease-free survival were calculated from the day of surgery to the day the patient succumbed or that of tumor relapse. The main clinical and pathological variables of the 98 patients are described in detail in Table I. The patients involved in the present study provided written informed consent. The investigation was approved by the Research Ethics Committee of Central South University, Changsha, China.

Immunohistochemistry. Immunohistochemical staining was performed using the PV-6001 Two-Step IHC Detection reagent following the manufacturer's instructions (Zhongshan Golden Bridge Biotechnology, Beijing, China). Briefly, antigen retrieval was carried out in 10 mmol/l citrate buffer (pH 6.0) for 10 min in a microwave oven at 750 W. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. Slides were incubated with Tiam1 rabbit polyclonal antibody (sc-872, dilution 1:50) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4˚C overnight, followed by the addition of HRP-labelled goat anti-rabbit polymers. Immunoreactive proteins were visualised with 3,3'-diaminobenzidine and counterstained with Mayer's hematoxylin. Negative control slides were probed with phosphate-buffered saline (PBS) under the same experimental conditions. The sections were independently evaluated and scored by two pathologists who were blinded to the clinical data of the patients. In case of disagreement, the sections were reviewed by a third person until a consensus conclusion was reached. The staining intensity was scored as 0 (no staining), 1 (weakly stained, light yellow), 2 (moderately stained, yellowish brown) and 3 (strongly stained, brown). The extent of staining was scored as 0 (absent), 1-5% (sporadic), 6-25% (local), 26-50% (occasional), 51-75% (majority) and 76-100% (large majority), according to the percentage of positively stained cells in the entire carcinoma-involved area. An intensity score of ≥2 with ≥50% of malignant cells with positive Tiam1 staining was considered high expression, and a <2 intensity score or <50% of malignant cells with positive staining was considered to be low expression of Tiam1 (12).

Cell culture and stable transfection. The human Hep-2 LSCC cell line, purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, was used in the present study. The cell line was grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 IU/ml penicillin and 100 IU/ml streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37˚C. The Tiam1 cDNA (C1199) plasmid cloned as a *Bam*HI/*Xho*I fragment in pcDNA3 containing a cytomegalovirus promoter and a hemagglutinin tag, a generous gift provided by Dr John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands), was used for stable transfection. Hep-2 cells ($3x10^5$ cells/well) were seeded into 6-well plates and allowed to grow at 50-70% confluence. The cells were transfected with Tiam1/C1199 plasmid and vector control using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 8 h, the original medium was replaced with fresh complete medium and the cells were subjected to the selection of stable clones in the presence of G418 (800 μg/ml) (Amresco, Solon, OH, USA) 48 h post-infection. The expression of Tiam1 was determined by western blotting after 3 weeks of G418 selection in RPMI-1640 medium containing 10% fetal bovine serum.

Western blotting. Total protein lysates, harvested from cells, were quantified by Bicinchoninic Acid Protein Assay kit (Beyotime, China). Total protein (40 μg) was separated by 8% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were incubated with rabbit polyantibody against Tiam1 (sc-872, dilution 1:100) (Santa Cruz Biotechnology Inc.) at 4˚C overnight. After washing, the cells were incubated with HRP-labelled goat anti-rabbit IgG for 2 h at room temperature. Bands were finally visualized by employing 3,3'-diaminobenzidine (Zhongshan Golden Bridge Biotechnology). Tiam1 protein expression levels were quantified by Quantity One software (Bio-Rad, Hercules, CA,USA). β-actin protein was determined using the specific antibody (BA2350, dilution 1:500) (Boster, China) as a loading control. All the experiments were carried out in triplicate.

MTT cell viability assay. The MTT assay was performed to evaluate the proliferation of transfected cells. The cells ($5x10^3$ cells/well) were plated in three 96-well plates in 200 μl of medium, and exposed to fresh media on alternate days. At 1, 2 and 3 day after plating, 20 μl MTT (5 mg/ml) (Sigma) was added to each well of one plate and after 4 h, the liquids were removed and 150 μl dimethyl sulfoxide (DMSO) was added. The optical density (OD) of each well was measured at 570 nm with a microplate reader. The cell growth curves were drawn according to time (x-axis) and OD value (y-axis).

Cell cycle analysis and cell apoptosis by flow cytometry. For cell cycle analysis, cells ($1x10^6$/ml) were washed with PBS at 4˚C twice and fixed in 70% alcohol for 24 h. Cell apoptosis was examined by flow cytometry using an Annexin V-FITC apoptosis detection kit (Invitrogen), following the manufacturer's instructions. The cells were washed twice in ice PBS and adjusted to a concentration of $1x10^6$/ml. The cells were subsequently gently mixed with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI), and incubated for 15 min at room temperature in the dark. After the addition of another 400 μl of 1X binding buffer, cell sorting analysis was detected on a FACSCalibur cytometer (Becton-Dickinson).

Cell migration and invasion assay. For the cell migration assay, the wound-healing assay was carried out. The cells ($5x10^5$ cells/well) were seeded in 6-well plates with complete medium and incubated for 12 h to until 90% confluence was reached. The cell monolayer was scratched with a 10 μl pipette tip, images were captured at 0 and 48 h after disruption in a phase-contrast microscope. Data presented are representative of three individual experiments.

The cell invasion assay was evaluated using 24-well Transwell chambers (Costar, Cambridge, MA, USA), according to the manufacturer's instructions. Briefly, Transwell with an 8-μm pore size insert membrane was coated with 100 μl Matrigel (BD Biosciences, Bedford, MA, USA) and incubated overnight. The cells ($3x10^4$ cells/well) in 100 μl of serum-free medium were seeded in the upper chamber of the Transwell, while the lower chamber was filled with 0.6 ml RPMI-1640 medium containing 15% fetal bovine serum to induce chemotaxis. After 24 h of incubation at 37˚C in humidified 5% CO_2 atmosphere, the non-migratory cells on the upper member surface were removed with a cotton tip, the migrated cells were fixed in 95% alcohol and stained with hematoxylin, and the number of migrated cells was counted in five randomly selected x400 power fields under the microscope. Results presented are representative of three individual wells.

Statistical analysis. Correlations between the expression of Tiam1 protein and clinicopathological parameters were calculated using the χ^2 test. Survival analysis was undertaken using the Kaplan-Meier method and curves were compared by the log-rank test. The Cox proportional hazards regression model was used in univariate and multivariate analysis to assess which factors were independent indicators for prognosis. Continuous variables were presented as mean ± SD. One-way ANOVA was used to compare the differences between the three cell groups. The SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. P<0.05 was considered to indicate a statistically significant result.

Results

Tiam1 expression in 98 cases of LSCC by immunohistochemistry. Positive Tiam1 immunostaining was predominantly diffusely distributed throughout the cytoplasm of tumor cells (Fig. 1). Among all the samples analyzed, 32 (32.7%) and 66 (67.3%) cases demonstrated a high and low Tiam1 protein expression. The χ^2 test was applied to assess the association between Tiam1 protein levels and various clinicopathological variables. As shown in Table I, Tiam1 overexpression was significantly associated with lymph-node metastasis (P<0.001), clinical stage (P=0.027), histological grade (P=0.020), and recurrence (P=0.003), respectively. However, no significant relationship was found between Tiam1 protein level and variables such as age, primary site and T stage (all P>0.05). According to the Tiam1 protein expression levels, 94 patients with follow-up (4 patients lost) were subdivided into two groups: 63 patients in the low Tiam1 expression group and 31 patients in the high Tiam1 expression group. The disease-free and overall survival rate in the 94 patients was 57.4 and 63.8%, respectively. Tiam1 expression in LSCC was significantly correlated with disease-free survival (P=0.001; Fig. 2A) and overall survival (P<0.001; Fig. 2B). The log-rank test also demonstrated that the survival time was significantly different between groups with a high and low expression of Tiam1 protein, indicating that a high level of Tiam1 was correlated with a shorter survival time. The Cox proportional hazards regression model was used to assess the effect of clinicopathological parameters and Tiam1 expression on the disease prognosis. In the univariate Cox analysis, lymph-node metastasis, clinical stage and Tiam1 expression were statistically correlated with prognosis. The multivariate Cox proportional hazards model analysis confirmed that lymph-node metastasis and Tiam1 expression were statistically significant, independent prognostic factors of outcomes in LSCC patients (Table II).

Table I. Correlations between Tiam1 expression and clinicopathological parameters in patients with laryngeal squamous cell carcinoma.

Clinicopathological variables	No. of pts.	Tiam1 expression		P-value[a]
		Low expression	High expression	
Age (years)				0.139
<58	41	31	10	
≥58	57	35	22	
Primary site				0.935
Glottic	74	50	24	
Supraglottic	24	16	8	
T stage				0.857
T1+T2	60	40	20	
T3+T4	38	26	12	
Lymph node metastasis				**<0.001**
N0	76	58	18	
N+ (N1, N2, N3)	22	8	14	
Clinical stage (TNM)				**0.027**
I-II	40	32	8	
III-IV	58	34	24	
Histological grade				**0.020**
G1	59	45	14	
G2+G3	39	21	18	
Recurrence[b]				**0.003**
Yes	40	20	20	
No	54	43	11	

[a]P≤0.05 was considered to be statistically significant (in bold). [b]Four patients were lost to follow up due to home moving or telephone number change. pts., patients; TNM, tumor-node-metastasis.

Upregulated expression of Tiam1 by plasmid Tiam1/C1199 cDNA in Hep-2 cells. To clarify the correlation of Tiam1 expression and LSCC metastasis, we employed the Tiam1/C1199 cDNA plasmid to improve the expression of Tiam1. Stable clones were isolated after selection with 800 μg/ml G418 for

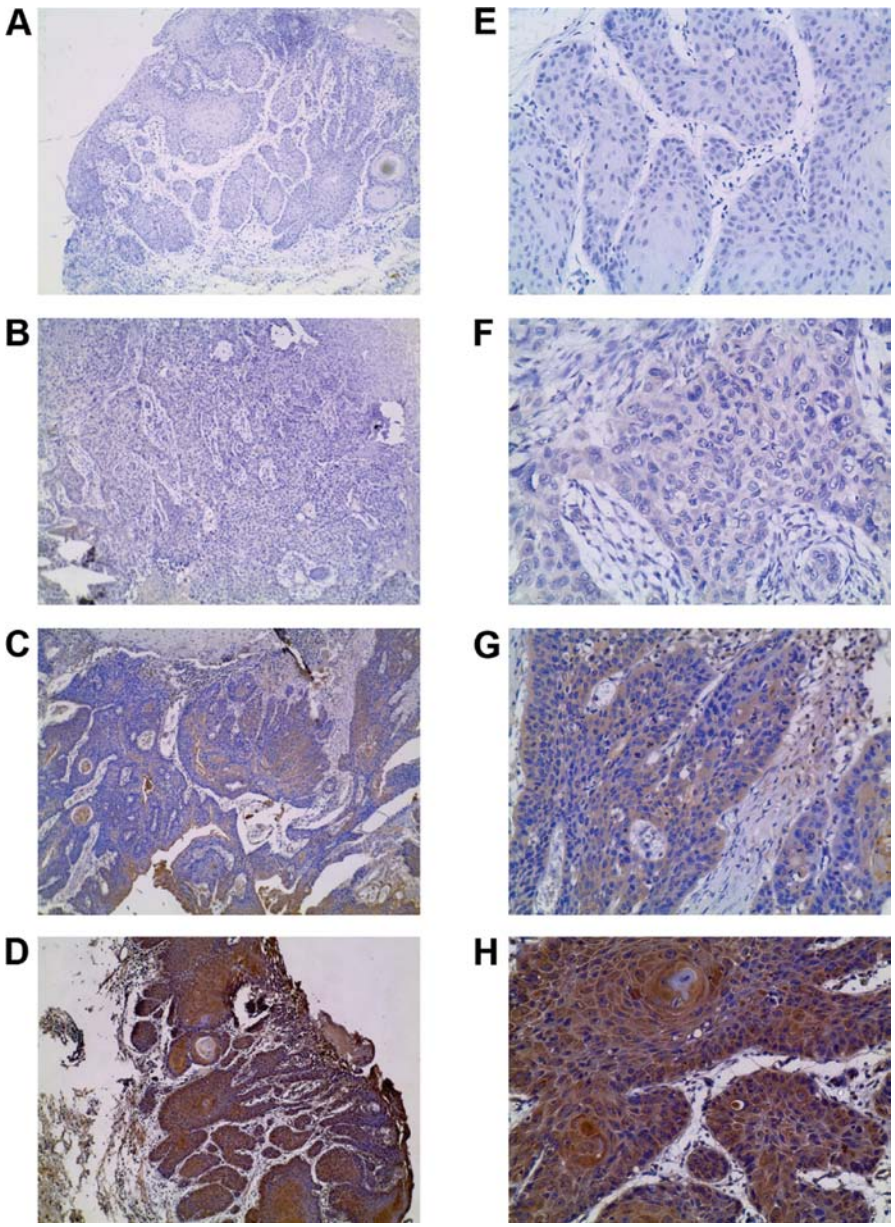

Figure 1. Immunohistochemistry of Tiam1 in human laryngeal squamous-cell carcinoma (LSCC) tissues. (A and E) Negative control of Tiam1 in LSCC tissues (primary antibody replaced with PBS). (B and F) Weak staining of Tiam1 in LSCC tissues. (C and G) Moderate staining of Tiam1 in LSCC tissues. (D and H) Strong staining of Tiam1 in LSCC tissues. (Original magnification, x100 in A-D, and x400 in E-H). Tiam1, T-lymphoma invasion and metastasis-inducing factor 1; PBS, phosphate-buffered saline.

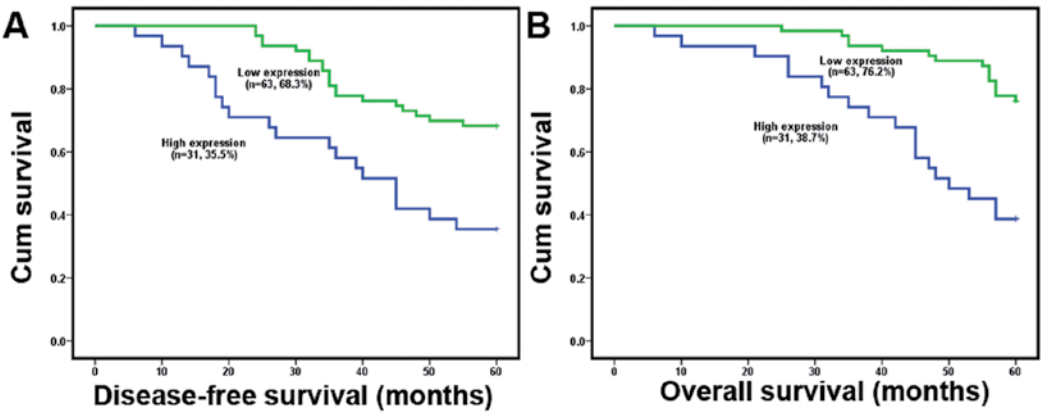

Figure 2. Kaplan-Meier survival curves showing (A) 5-year disease-free and (B) overall survival rate of patients with a high or low Tiam1 expression. Log-rank test demonstrated that patients with laryngeal squamous-cell carcinoma in the high Tiam1 expression group had a shorter disease-free survival rate (P=0.001) and poorer overall survival rate (P<0.001) compared with those in the low Tiam1 expression group. Tiam1, T-lymphoma invasion and metastasis-inducing factor 1.

Table II. Cox proportional hazards regression model analysis of overall survival.

Clinicopathological variables	Univariate analysis		Multivariate analysis	
	Exp (B)	P-value	Exp (B)	P-value
Age (years) (<58/≥58)	1.746	0.129	0.624	0.300
Primary site (glottic/subglottic)	1.010	0.980	1.089	0.833
T stage (T1+T2/T3+T4)	0.489	0.066	3.424	0.142
Lymph node metastasis (N0/N+)	6.757	**<0.001**	29.241	**0.001**
Clinical stage (I-II/III-IV)	1.315	0.044	0.145	0.066
Histological grade (G1/G2+G3)	1.225	0.560	1.437	0.346
Tiam1 expression (high/low)	0.266	**<0.001**	0.401	**0.020**

P-value in bold were statistically significant.

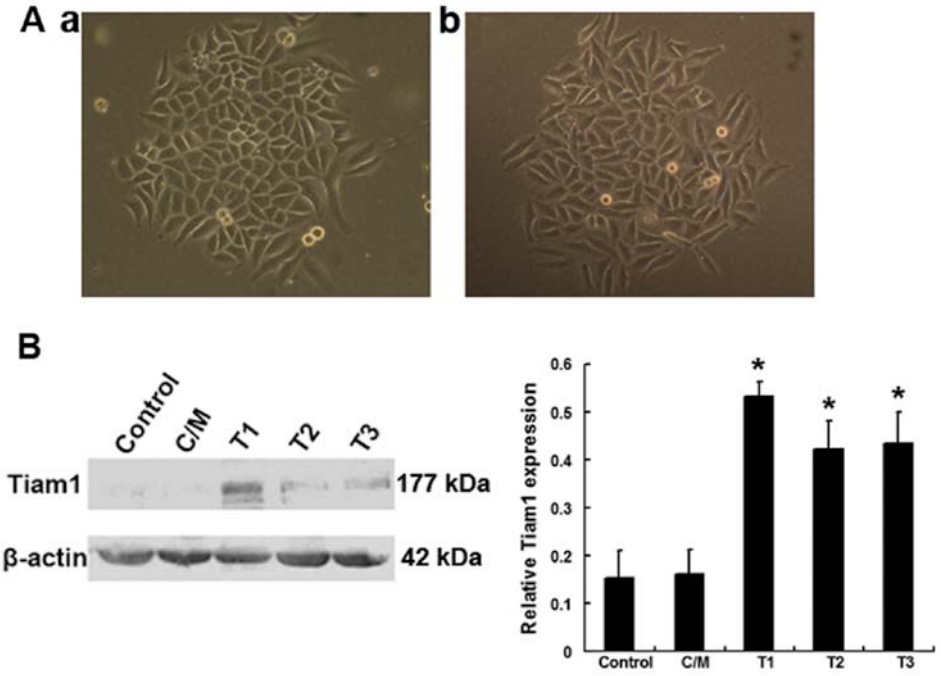

Figure 3. Overexpression of Tiam1 by transfection of Tiam1/C1199 plasmid in Hep-2 cell line. (A) Positive and negative cell clones that transfected different plasmid were selected by G418 (magnification, x100). (a, Negative cell clones; b, positive cell clones). (B) Upregulation of Tiam1 detected by western blotting. (*P<0.001, T1, T2, T3 vs. control, C/M) (control, Hep-2 cell group; C/M, Hep-2/Mock cell group; T1, T2 and T3, Hep-2/Tiam1 cell group). Tiam1, T-lymphoma invasion and metastasis-inducing factor 1.

3 weeks. We randomly selected three positive cell clones that were transfected with Tiam1/C1199 cDNA (T1, T2 and T3) plasmid, and one negative cell clones that was transfected with vector control (C/M). Western blotting was carried out to assess the efficiency in the upregulation of Tiam1 expression. As shown in Fig. 3, Tiam1/C1199 cDNA was successfully transfected into the LSCC cells, and the level of Tiam1 expression was obviously upregulated (P<0.001). Moreover, we selected T1 cell clones for the subsequent experiments *in vitro*. The Hep-2 cells-transfected Tiam1/C1199 cDNA plasmid and vector control were termed, for convenience, as Hep-2/Tiam1 and Hep-2/Mock, respectively.

Upregulated Tiam1 expression has no effect on proliferation of Hep-2 cells in vitro. To determine the effect of Tiam1 on the proliferation of Hep-2 cells *in vitro*, an MTT assay was performed and a cell growth curve was obtained. As shown in Fig. 4, no difference in cell growth between Hep-2/Tiam1 and Hep-2 and Hep-2/Mock control groups was identified (P>0.05).

Upregulated Tiam1 expression has no effect on cell cycle but decreases apoptotic rate of Hep-2 cells in vitro. To further elucidate the effect of upregulated Tiam1 on cell growth in Hep-2 cells, flow cytometry was carried out to monitor the cell cycle and apoptotic changes. The results demonstrated no difference between Hep-2/Tiam1 and the Hep-2 and Hep-2/Mock control groups in the G1 phase of the cell cycle (66.02±3.48 vs. 66.74±1.54, 67.00±2.22) (P>0.05), in the S phase of the cell cycle (21.15±1.50 vs. 19.65±1.61, 17.83±3.63) (P>0.05) and in the G2 phase of the cell cycle (11.82±3.92 vs.

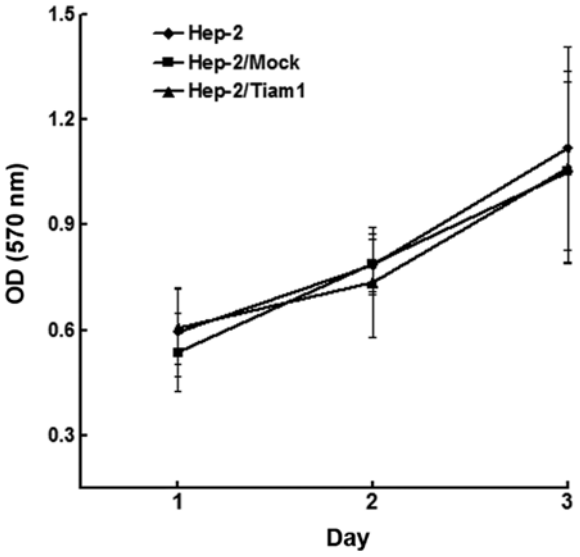

Figure 4. Effect of Tiam1 overexpression on the proliferation of Hep-2 cells. Cell proliferation was measured by the MTT assay every 24 h for 3 days. Tiam1, T-lymphoma invasion and metastasis-inducing factor 1.

13.66±2.08, 15.40±1.50) (P>0.05) (Fig. 5A and B). However, when compared with the control groups, the apoptotic rate of Hep-2/Tiam1 was significantly decreased (2.50±1.33 vs. 5.61±1.08, 5.83±1.02) (P=0.021) (Fig. 5C and D). These results demonstrated that upregulated Tiam1 expression has no effect on cell growth, but decreases the apoptotic rate of Hep-2 cells.

Upregulated Tiam1 expression promotes the migration and invasion of Hep-2 cells in vitro. To determine the effect of Tiam1 on Hep-2 cell migration and invasion, wound-healing assay and Transwell invasion chambers were performed. As shown in Fig. 6A and C, upregulated Tiam1 expression can lead to significantly increased migration (Hep-2/Tiam1 group, migration distance 147.00±26.06 vs. Hep-2, Hep-2/Mock control groups, migration distance 83.33±23.18 and 96.33±16.01; P=0.028) and invasion (68.33±6.66 vs. 43.00±8.89 and 45.00±7.55; P=0.013) of Hep-2 cells. These results clearly demonstrate that Tiam1 plays a functional role in mediating cell migration and invasion in LSCC, which are also the key factors of LSCC malignant progression and metastasis.

Discussion

The oncogenic potential of Tiam1, the specific GEF for the Rho-like GTP-binding proteins, is achieved by the activation of Rac signaling pathway (18). Tiam1-Rac signaling is capable of stimulating the p38 mitogen-activated protein kinase pathway, c-Jun N-terminal kinase pathway, and extracellular signal-regulated kinase pathway (19-21), which are considered to be associated with the regulation of gene transcription. Tiam1 is involved in modulating alteration of the actin cytoskeleton, cell migration (22-24), tumor microenvironment (25) and cell polarity (26), which are considered relevant to tumor progression.

The relationship between Tiam1 overexpression and various types of cancer has been previously identified (7-14). However, to the best of our knowledge, few studies focusing on LSCC are available. Consequently, in the present study, we examined Tiam1 expression in LSCC tissue samples and assessed its clinical prognostic value. Furthermore, the role of Tiam1 in the proliferation, apoptosis, migration and invasion of LSCC was elucidated *in vitro*.

In the present study, the results of 98 LSCC tissue samples investigated by immunohistochemistry demonstrated that a high Tiam1 expression was associated with lymph-node metastasis, recurrence, histological grade and clinical stage, which indicated the importance of Tiam1 in LSCC progression and metastasis. Additionally, the results of the present study show that Tiam1 overexpression was correlated with shorter disease-free and overall survival, and that Tiam1 may provide independent information to predict the clinical outcome of LSCC patients. The results of the Cox analysis revealed that Tiam1 expression and lymph-node metastasis were significant and independent prognostic factors involved in overall survival.

In the subsequent experiments, Tiam1/C1199 plasmid was successfully transfected to upregulate the expression of Tiam1 in LSCC cells. The MTT cell viability assay revealed that there was no significant effect on cell proliferation when LSCC cells were transfected with Tiam1/C1199 plasmid. Cell cycle analysis by flow cytometry also revealed that there was no difference in the G1, S and G2 phase of the cell cycle in the transfected cells. These results indicated that the upregulation of Tiam1 had no effect on the proliferation of LSCC cells. As mentioned in the immunohistochemistry results, Tiam1 overexpression was not associated with T stage of LSCC patients. Thus, the results of the MTT assay and cell cycle analysis *in vitro* are in concordance with the results obtained from immunohistochemistry. However, the critical role of Tiam1 in the proliferation of LSCC cells is discordant with its suggested roles in other reported cancers, such as colorectal cancer and hepatocellular carcinoma (27,28). The discrepancy in these results is associated with the cell type-specific of Tiam1 in cell proliferation.

Cell migration and invasion play critical roles in cancer metastasis. Our *in vitro* data clearly indicated that upregulation of the Tiam1 expression increased the ability of migration and invasion of LSCC cells. Moreover, our results are in concordance with Tiam1 as identified in other reported cancers (27-29). Anoikis is reported to act as a physiological barrier to metastasis, however, resistance to anoikis may allow survival of cancer cells during metastasis (30). Cell apoptosis by flow cytometry in the present study showed that the apoptotic rate was decreased when LSCC cells were transfected with Tiam1/C1199 plasmid. Thus, we suggest that upregulation of Tiam1 expression can increase the ability of metastasis through a decrease of the LSCC cell anoikis rate. However, the underlying mechanisms regarding how Tiam1 regulates tumor metastasis have yet to be adequately elucidated.

In summary, results of the present study have demonstrated that the overexpression of Tiam1 was common in LSCC, which suggested that Tiam1 may play a critical role in inducing the tumorigenesis of LSCC. We also found that the upregulation of Tiam1 expression increased LSCC cell metastatic ability *in vitro*, suggesting that Tiam1 is a valuable therapeutic target for patients with LSCC. Future studies

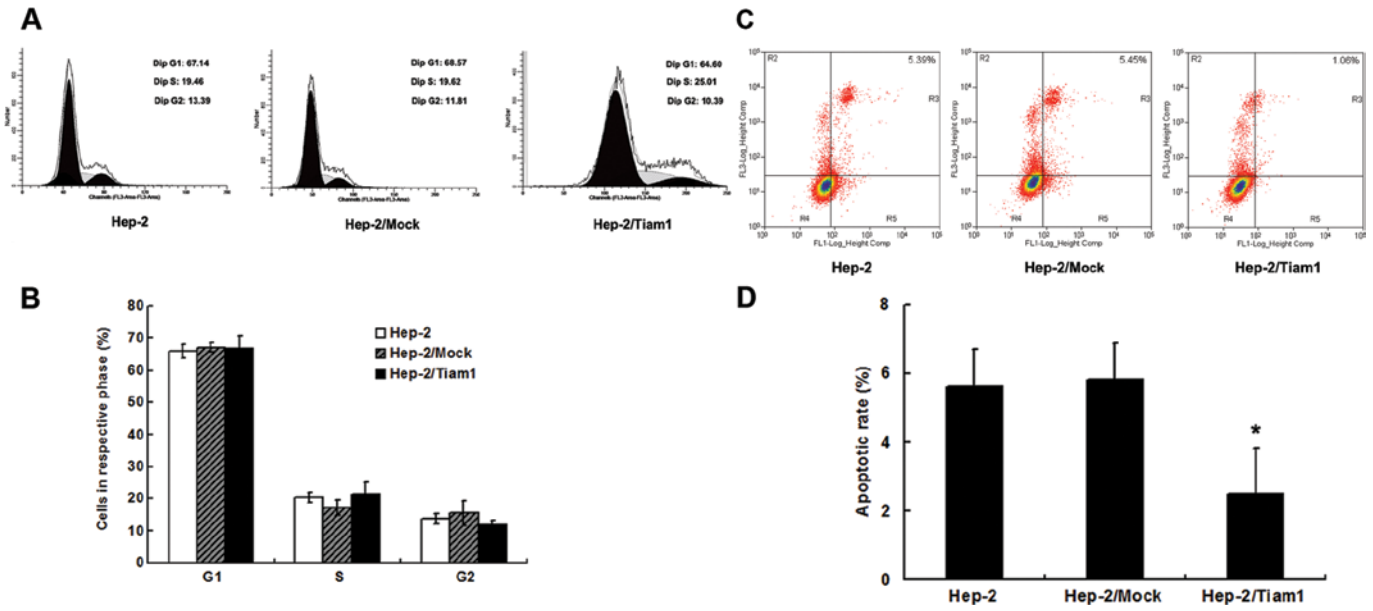

Figure 5. Effect of Tiam1 overexpression on cell cycle distribution and cell apoptosis of Hep-2 cells *in vitro*. (A) Representative histograms showing cell cycle profiles of Hep-2, Hep-2/Mock and Hep-2/Tiam1 cells. The cells were stained with propidium iodide (PI) and analyzed by flow cytometry. (B) Proportion of cells in various phases of cell cycle. (C) Representative histograms showing cell apoptosis of Hep-2, Hep-2/Mock and Hep-2/Tiam1 cells. Cells staining negative for PI but positive for Annexin V-FITC were considered to have undergone apoptosis. (D) Different apoptotic rates of various groups. (*P<0.05, Hep-2/Tiam1 vs. Hep-2, Hep-2/Mock). Tiam1, T-lymphoma invasion and metastasis-inducing factor 1.

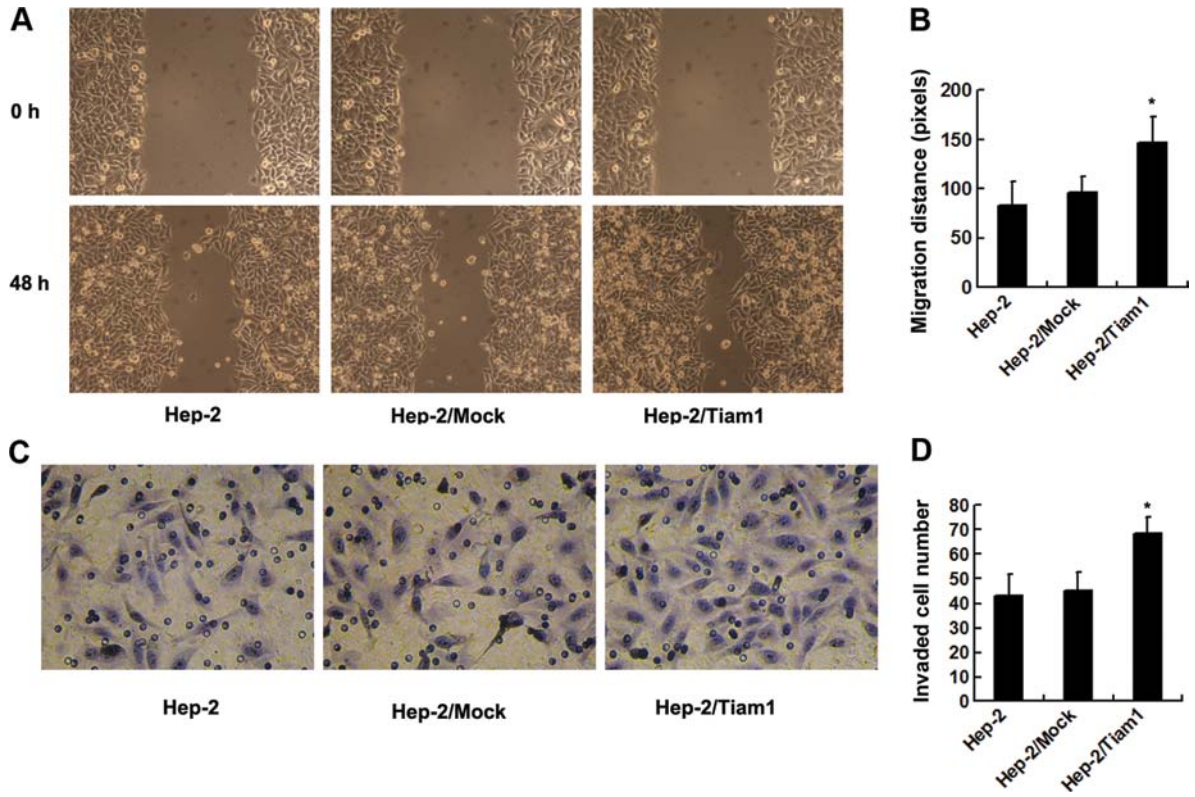

Figure 6. Effect of Tiam1 overexpression on migration and invasion of Hep-2 cells *in vitro*. (A) Wound-healing assay demonstrated that the overexpression of Tiam1 promoted the migration of Hep-2 cells. Images were captured at 0 and 48 h after wound induction at x100 magnification. (B) Graphical representation of the distance of migration of Hep-2 cells. (C) Transwell invasion assay showed that overexpression of Tiam1 promoted the invasion of Hep-2 cells. Images were captured at x200 magnification. (D) Graphical representation of the numbers of invaded Hep-2 cells/microscopic field. (*P<0.05, Hep-2/Tiam1 vs. Hep-2, Hep-2/Mock). Tiam1, T-lymphoma invasion and metastasis-inducing factor 1.

should focus on the effect of Tiam1 overexpression *in vivo* and the precise mechanisms of Tiam1 in metastasis, which may lead to the identification of new techniques for the prevention of the progression of LSCC patients.

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