ASAP1 mediates the invasive phenotype of human laryngeal squamous cell carcinoma to affect survival prognosis

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Abstract. ASAP1 helps regulate cellular structures such as actin cytoskeletal remodeling and focal adhesions that have a pivotal function in tumor progression. Overexpression of ASAP1 has proven to be a malignant indicator for a variety of tumors. To further determine the potential involvement of ASAP1 in laryngeal squamous cell carcinoma (LSCC), we evaluated the expression levels of ASAP1 by quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) and immunohistochemistry in tissue samples of 64 LSCC patients. We then analyzed and correlated the results with clinicopathological features. Furthermore, we used small interfering RNA (siRNA) to inhibit ASAP1 expression in vitro. The potential function of ASAP1 in invasiveness was evaluated in the Hep-2 LSCC cell line. Kaplan-Meier method was utilized to determine the association of ASAP1 expression with survival of patients. We showed that ASAP1 was upregulated in primary LSCC tumors and was correlated with lymph node metastasis and clinical tumor stage. Similarly, higher levels of ASAP1 were detected in the Hep-2 cell line compared to the 16 human bronchial epithelial (16HBE) cell line. ASAP1 expression was downregulated by lentiviral vector transfection containing siRNA in vitro. The invasive potential of these cells was found to be significantly suppressed, while expression levels of Rac1 and Cdc42 positively correlated with the inhibition of ASAP1 expression. In Kaplan-Meier overall survival curves, higher ASAP1 mRNA levels were found to be associated with a shorter progression-free survival trend. Based on these results, ASAP1 appears to contribute to the malignant mechanism of LSCC and may represent a significant prognostic marker for LSCC patients.

Introduction

ASAP1, also known as AMAP1, DDEF1, DEF1 or centaurin β4, is an ADP-ribosylation factor GTP-ase activating protein that induces the hydrolysis of GTP molecules (1-3). ASAP1 contains multiple domains, including BAR, Ankyrin repeat, proline-rich, SH3 and pleckstrin homology (PH) domains. The N-terminal BAR domain induces membrane tubulation and can function as a protein binding site (4,5), while the Ankyrin and SH3 domains facilitate signaling and interactions by ASAP1 with focal adhesion kinase (FAK), CD2AP, Src and paxillin (6-10). Using its pleckstrin homology domain, ASAP1 also regulates ADP-ribosylation factors (ARFs) which cycle between GDP and GTP states (11).

Rac and Cdc42 are small GTP-binding proteins of the Rho family, a subset of the Ras superfamily (21). Rac1 and Cdc42 have been well-characterized, and similar to the other GTPases, they cycle between a GTP-bound and a GDP-bound form. It is hypothesized that their activity is regulated by a combination of GTPase-activating proteins (GAPs), guanine nucleotide exchange factors and guanine nucleotide dissociation inhibitors (GDIs). As such, these small GTP-binding proteins serve as molecular switches within numerous signal transduction pathways and regulate a diverse set of cellular functions, including control of cell morphology, cell cycle

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progression, cell migration and invasion, cell growth and proliferation, actin dynamics and the transcriptional activation of apoptotic signaling (22-29). However, it is currently unknown whether ASAP1 affects the activation of Rac and Cdc42 in LSCC.

Carcinoma of the larynx is the most common malignant neoplasm of the head and neck and is associated with a high rate of mortality. Moreover, many of these patients are diagnosed with squamous cell carcinomas. Currently, the treatment options for laryngeal carcinoma include radical laryngectomy and conservative chemotherapy or radiotherapy. However, despite advances in these treatment methods, the mortality rate of advanced stage laryngeal cancer remains largely unchanged. Metastasis of primary laryngeal cancers also contributes to the high mortality rates associated with these tumors, and the mechanisms associated with this process remain largely uncharacterized. Thus, mechanistic studies of LSCC metastasis continue to be an important area of research, as well as the identification of more effective biological markers for the diagnosis and prognosis of LSCC.

Currently, it remains unknown whether ASAP1 plays a role in laryngeal carcinoma. Therefore, in the present study, expression of ASAP1 mRNA was detected in laryngeal carcinoma tissues and matched non-tumor tissues using real-time PCR and immunohistochemistry assays. The potential for ASAP1 to regulate the invasive phenotype of Hep-2 cells in vitro was also evaluated using a lentivirus vector system. Finally, the prognostic value associated with ASAP1 mRNA levels was analyzed.

Materials and methods

Patients and specimens. A retrospective review of 80 adult patients with pathologically confirmed primary LSCC was carried out. Between 2006 and 2007, these patients underwent a partial or total laryngectomy at the Department of Otorhinolaryngology, at the Second Affiliated Hospital of Harbin Medical University. Cancer tissues and corresponding adjacent normal tissues were collected during surgery and were subsequently fixed in formalin and embedded in paraffin (FFPE) according to standard pathology protocols. Patient characteristics for this cohort are listed in Table I. Following treatment, patients underwent follow-up through December 2007, with endpoints classified as: surviving without LSCC, death due to primary recurrent LSCC, death due to other causes and follow-up status unknown. Surviving patients were confirmed by phone or by checking census records. For 64/80 patients, complete clinicopathological data and tumor specimens in a good state of preservation were available. Therefore, these patients were examined in the present study. The study protocol used was in accordance with the institutional guidelines for human research and was approved by the ethics committee.

Lentiviral vector system. A recombinant lentivirus was generated that contained siRNA designed to target the human mRNA sequence of ASAP1 (siRNA-ASAP1) (5'-GACCAG AUCUCUGUCUGGAGUUC-3' and 5'-UGAACUCCGA GACAGAGUCUGGUC-3'), and these were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). A control lentivirus was also generated. To monitor transfection efficiency, both lentiviruses included a green fluorescent protein (GFP) cassette. The lentiviruses containing siRNAs targeting ASAP1 and GFP control lentiviruses were titered to 10^5 TU/ml preparations and polynbrene (8 mg/ml) were added to each well. The cells were incubated at 37°C for 12 h. The supernatant from each well was then removed, and DMEM containing 10% FBS and 1%

Table I. Levels of ASAP1 mRNA and the clinicopathological characteristics of the LSCC patients examined.

<table>
<thead>
<tr>
<th>Patient characteristics (No. of patients)</th>
<th>Levels of ASAP1 mRNA (T/N ratio)a</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=42)</td>
<td>4.42±0.24</td>
<td>0.31</td>
</tr>
<tr>
<td>Female (n=22)</td>
<td>4.49±0.29</td>
<td></td>
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<tr>
<td>T classification</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>T1-2 (n=43)</td>
<td>4.40±0.26</td>
<td></td>
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<tr>
<td>T3-4 (n=21)</td>
<td>4.48±0.31</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Negative (n=39)</td>
<td>4.32±0.26</td>
<td></td>
</tr>
<tr>
<td>Positive (n=25)</td>
<td>4.58±0.22</td>
<td></td>
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<tr>
<td>Differentiation</td>
<td></td>
<td>0.32</td>
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<tr>
<td>G1 (n=47)</td>
<td>4.40±0.25</td>
<td></td>
</tr>
<tr>
<td>G2-G3 (n=17)</td>
<td>4.48±0.34</td>
<td></td>
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<tr>
<td>Clinical stage</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>I-II (n=36)</td>
<td>4.44±0.26</td>
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<tr>
<td>III-IV (n=28)</td>
<td>4.59±0.22</td>
<td></td>
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<tr>
<td>Patient age (years)</td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>≤60 (n=24)</td>
<td>4.44±0.23</td>
<td></td>
</tr>
<tr>
<td>≥60 (n=40)</td>
<td>4.45±0.26</td>
<td></td>
</tr>
<tr>
<td>ASAP1 mRNA levels</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Low (n=32)</td>
<td>4.22±0.21</td>
<td></td>
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<tr>
<td>High (n=32)</td>
<td>4.63±0.16</td>
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</tbody>
</table>

aT/N ratio, tumor tissue/normal tissue (T/N) ratio: the fold-change in ASAP1 mRNA levels relative to the human U6 gene in tumor tissues and matched normal tissues according to the 2^(-ΔΔCt) method. Values indicated are the means ± standard deviation for each group.

Cell culture and transfection. The human LSCC cell line, Hep-2, was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Shanghai Shenggong Co., Ltd., Shanghai, China) and were maintained at 37°C under a humidified atmosphere containing 5% CO2. Hep-2 cells in the logarithmic phase of growth were seeded in 6-well plates at a concentration of 1x10^5 cells/well for transfection. After 12 h, when cells reached ~40-50% confluency, 1 ml of complete medium containing lentivirus (10^5 TU/ml) preparations and polynbrene (8 mg/ml) were added to each well. The cells were incubated at 37°C for 12 h. The supernatant from each cell was then removed, and DMEM containing 10% FBS and 1%
penicillin-streptomycin was added. After 24 h, the culture medium was replaced with fresh DMEM. Seventy-two hours post transfection, the mean percentage of GFP-positive cells observed in each well was calculated from three random fields of view (FOV) at x200 magnification using a fluorescence microscope (IX70; Olympus, Tokyo, Japan).

RNA isolation and quantitative real-time PCR. RNA was isolated from well-preserved FFPE tissues using a High Pure RNA Paraffin kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. RNA was also isolated from Hep-2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were determined by absorbance readings at 260 nm, while RNA purity was evaluated according to the OD$_{260}$/OD$_{280}$ absorption ratios obtained.

cDNA was reverse transcribed using an All-in-One™ miRNA qPCR Detection kit (GeneCopoeia, Rockville, MD, USA). The reverse transcription reactions were performed at 37°C for 60 min, then at 70°C for 5 min. Real-time PCR was performed using a SYBR-Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) and a 7500 Fast Real-Time PCR System (Applied Biosystems). Reactions were incubated at 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 15 sec. Expression data were normalized to human U6 gene expression data obtained in parallel as an external reference using the 2$^{-ΔΔCt}$ method. The forward and reverse primers used for detection of ASAP1 included: 5'-TG TAGTCTTACTTTGAAGGATGGACC-3' and 5'-CCCTC CCAGGCCAATCCT-3', respectively, and were synthesized by GeneCore BioTechnologies Co., Ltd. (Shanghai, China). Each reaction was performed in triplicate.

Invasion assay. Seventy-two hours post-transduction, cells (2x10$^5$) were resuspended in 200 µl serum-free medium and were plated in the upper chambers of Boyden chambers (24-well, 8-mm pores) coated with Matrigel (Becton-Dickinson Labware, NJ, USA). The lower chambers contained 1 ml medium containing 10% FBS as a chemoattractant. Untreated Hep-2 cells were also plated as controls. After 24 h, cells were mechanically removed from the upper side of the filters, while cells that had migrated to the lower side of the filters were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E). The number of cells present in three x200 microscopic fields for each well were averaged and reported. Five independent experiments were performed.

Western blotting. Cells from the siRNA lentivirus group, the GFP control lentivirus group, and untreated Hep-2 cells were harvested 72 h post-transfection and incubated in cell lysis buffer for 30 min on ice. Cell lysates were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to polyvinylidene fluoride (PVDF) membranes. After incubating the membranes in 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used included rabbit anti-human ASAP1 (1:500; Abcam, Cambridge, MA, USA), rabbit anti-human rac1 (1:100; Abnova, Taipei, Taiwan), and rabbit anti-human Cdc42 (1:200; Bioss, Beijing, China). The membranes were then washed with TBTS and incubated with species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at 37°C. β-actin served as a loading control, and bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Three independent experiments were performed.

Immunohistochemistry. FFPE specimens were sequentially sectioned (4 µm each). After deparaffinization and rehydration, sections were treated with 0.3% H$_2$O$_2$, and then were incubated with 10% normal goat serum. Antigen retrieval was performed using EDTA (pH 8.0) at 100°C for 20 min. Sections were then washed, and incubated with the primary antibodies at 4°C overnight. Sections were subsequently incubated at 37°C for 45 min before being washed and incubated with secondary antibodies at room temperature. After 1 h, sections were washed and incubated with diaminobenzidine tetra-chloride for 10 min. Sections were then counterstained with haematoxylin. Negative controls were prepared in parallel and were incubated with PBS instead of the primary antibodies.

ASAP1 scoring. ASAP1 immunoreactivity was evaluated in blinded analyses performed by two pathologists. Staining intensity was graded from 1 to 4, and these scores indicated an absence of staining (receiving a score of 1) up to strong staining of ASAP1 (receiving a score of 4). Mean score values for each sample were reported.

Statistical analysis. The SPSS statistical software package was used for all calculations. An independent t-test was used to analyze differences in ASAP1 mRNA levels, ASAP1 protein scoring, and the invasive phenotype between groups. Overall survival (OS) was defined as the time period from the date of surgery to the date of death due to LSCC or other causes, or to the end of the study (2011-12-31). The log-rank test was used to evaluate the association of risk factors with time-to-event endpoints. OS curves were also calculated using the Kaplan-Meier method. A P-value <0.05 was considered to indicate a statistically significant result.

Results

ASAP1 is overexpressed in human LSCC both in vivo and in vitro. Real-time PCR was used to determine the expression

![Graph](image)

Figure 1. Expression of ASAP1 mRNA in Hep-2 cells vs. 16HBE cells. Levels of ASAP1 mRNA were 3.6-fold higher in the Hep-2 cells compared to the 16HBE cells as detected by real-time RT-PCR. *P<0.05.
status of ASAP1 mRNA for both FFPE LSCC tissue samples and matched normal tissue samples obtained from 64 patients diagnosed with LSCC. For the LSCC samples, the mean mRNA level for ASAP1 was 4.45-fold higher than that of the corresponding matched samples (range, 3.76-4.97; median, 4.41) (P<0.05). Based on these data, the LSCC patients were divided into a low expression group (ASAP1 mRNA level ≤4.41) and a high expression group (ASAP1 mRNA >4.47). A similar observation was made in vitro, where the Hep-2 cells exhibited higher levels of ASAP1 mRNA than the 16HBE cells (Fig. 1).

Consistent with the mRNA levels detected for ASAP1, immunohistochemistry assays detected stronger expression of ASAP1 in tissue sections from the high expression group compared to tissue sections from the low expression group. Moreover, the mean ASAP1 score for the high expression group (2.63±0.87) was significantly higher than that of the low expression group (2.19±0.82) (P<0.05). ASAP1 expression was also detected in a subset of the normal tissues stained, although the staining intensity was much weaker compared to that observed for the cancer tissues, particularly in the cytoplasm (Fig. 2).

Levels of ASAP1 mRNA and clinicopathological factors. We analyzed the relationships between levels of ASAP1 mRNA in the LSCC tumors and the clinical data from these patients (Table 1). There were no obvious differences in the mRNA levels associated with gender, tumor grade and patient age. However, significantly higher levels of ASAP1 mRNA present in LSCC tissues were associated with lymph node metastasis and clinical tumor stage. For example, patients with higher ASAP1 mRNA levels tended to have an advanced clinical stage of LSCC or lymph node metastasis. These results suggest that upregulated expression of ASAP1 mRNA correlates with the progression of LSCC and an invasive phenotype.

Targeting of ASAP1 mRNA in the Hep-2 cells. In order to investigate the biological function of ASAP1 in LSCC, recombinant lentiviruses containing specific siRNA designed to target the human mRNA sequence of ASAP1, as well as a GFP cassette, were created. Seventy-two hours after Hep-2 cells were transduced with siRNA-ASAP1 and GFP control lentiviruses, >80% of the Hep-2 cells were observed to express GFP, indicating the efficiency and stability of the transductions performed (Fig. 3A). These cells were then subjected to
quantitative real-time PCR. Levels of ASAP1 mRNA were found to be considerably lower in the cells transfected with the siRNA-ASAP1 lentivirus compared to the GFP control and untreated cells, thereby validating the use of this lentiviral vector system for studies of ASAP1 (P<0.05) (Fig. 3B).

Downregulation of ASAP1 suppresses the invasion of Hep-2 cells in vitro. To investigate whether ASAP1 contributes to the invasive phenotype of Hep-2 cells, invasion assays were performed using 24-well Boyden chambers coated with Matrigel. As shown in Fig. 4, the number of siRNA-ASAP1-treated Hep-2 cells exhibiting an invasive phenotype 72 h after transfection (35.8±4.49) was less than that observed for GFP control-treated Hep-2 cells (44.8±5.07) and untreated Hep-2 cells (46.4±5.32) (P<0.05). These data strongly suggest that downregulation of ASAP1 may mediate a reduction in the invasiveness of laryngeal carcinoma cells.

ASAP1 expression positively correlates with Rac1 and Cdc42 expression. Western blotting was used to measure expression levels of ASAP1, Rac1 and Cdc42 in the Hep-2 cells transfected with siRNA-ASAP1 and GFP control lentiviruses. When Hep-2 cells were transfected with the siRNA-ASAP1 lentivirus, lower levels of ASAP1 were detected compared to cells transfected with the GFP control lentivirus or the untreated cells (P<0.05). In these same samples, lower levels of (B) Rac1 and (C) Cdc42 expression were detected in the siRNA-ASAP1 samples but not in the GFP control cells and untreated Hep-2 cells. Detection of β-actin was used as a loading control. *P<0.05.

Prognostic significance of ASAP1 mRNA levels. Of the 64 patients examined, 26 died during the follow-up period. Depending on the levels of ASAP1 that were detected, the 3-year survival probability was 65.6 and 81.3% for cases with high vs. low levels of ASAP1 mRNA, respectively. The 5-year survival probability for the same groups was 43.8 and 75.0%, respectively (P<0.05) (Fig. 6). Taken together, these results suggest that patients with LSCC tumors that express high levels of ASAP1 have a poor prognosis and will experience a shorter survival period compared with patients presenting with LSCC tumors that express low levels of ASAP1.
Despite advances achieved in the diagnosis and treatment of LSCC in recent years, the long-term survival rate for LSCC, and especially the survival rate for advanced stages of LSCC, have not significantly improved. In addition, metastasis of LSCC still represents a significant challenge, and contributes to the mortality rates reported for LSCC patients each year. While the expression of various genes has been correlated with the development of metastatic LSCC, and some of these have the potential to serve as markers of tumor progression in the clinic (30,31), the mechanistic details for LSCC remain to be determined.

Previously, Miyata et al (32) identified a region in the 5’-UTR of AMAPI that exhibits a significant internal ribosome entry site (IRES) activity in differentiated U937 cells. Moreover, this activity appears to be necessary for enhanced AMAPI expression in this cell line. However, this IRES-dependent mechanism for AMAPI expression may not be conserved in all tumor cells. Currently, the role of AMAPI in LSCC remains largely uncharacterized. In colorectal tumors, AMAPI expression is strongly upregulated (16), and Lin et al (18) reported that levels of AMAPI were elevated in 80% of primary prostate cancer samples. The results of the present study are consistent with these results, and further demonstrated that both mRNA and protein levels of AMAPI were upregulated in LSCC tissues compared to paired normal tissues. A similar observation was made in vitro for the Hep-2 and 16HBE cell lines. Based on these findings, it is hypothesized that high levels of AMAPI expression contribute to LSCC.

Cell dissemination is a complex cell motility phenomenon that requires the coordination of protrusion, chemotaxis, invasion and contractile activities of cancer cells in order to achieve directed cell migration. Roles for ASAP1 in actin cytoskeleton remodeling and local adhesion have previously been demonstrated (6,12). More recently, ASAP1 has been implicated in mediating the invasive phenotypes of tumor cells (33,34). In the present study, Boyden chamber assays were used to characterize the role of ASAP1 in relation to the invasive phenotype of LSCC in vitro, and overexpression of ASAP1 was found to promote the invasive activity of Hep-2 cells. Similarly, ASAP1 has been shown to mediate the invasion and metastasis of breast cancer cells via the EGFR-GEP100-ArF6-AMAPI signaling pathway, or in cooperation with CIN85 or Rab5c (35-37). Overexpression of ASAP1 has also been associated with increased invasion and metastatic potential of high-grade uveal melanomas (17). Therefore, the findings of the present study are consistent with the results obtained using other tumor models, and further support a role for ASAP1 in tumor invasion.

Directed cell migration involves modulation of the actin cytoskeleton by Rho GTPases. In fibroblasts, Rac proteins regulate the formation of lamellipodia and membrane ruffles, as well as the subsequent formation of stress fibers (38,39). In contrast, Cdc42 plays a key role in the formation of filopodia at the cell periphery, and this is followed by the formation of lamellipodia and membrane ruffles (40). When ASAP1 was downregulated in Hep-2 cells using an siRNA targeting ASAP1 lentivirus, cell invasion was inhibited. Furthermore, reduced ASAP1 expression also positively correlated with the protein levels detected for Rac1 and Cdc42. Taken together, these results suggest that decreased expression of Rac1 and Cdc42 may also contribute to the invasive phenotype of Hep-2 cells.

To determine whether ASAP1 expression levels affect the prognosis of laryngeal cancer patients, the median level of ASAP1 mRNA that was identified in 64 LSCC patients was used to establish a high ASAP1 expression level group and a low ASAP1 expression level group. The former was associated with a higher mortality rate, as well as a higher rate of relapse. To the best of our knowledge, this is the first study to investigate the effect of ASAP1 expression on the recurrence and mortality of LSCC. Given the small sample size of this study, additional studies are needed to validate the present results. However, the results of the present study do highlight the potential for ASAP1 to be considered as a risk factor for human laryngeal carcinoma.

Based on the results obtained, ASAP1 appears to have an oncogenic role in the metastasis of laryngeal tumors. Therefore, it is hypothesized that ASAP1 mRNA and ASAP1 represent therapeutic targets and prognostic biomarkers that should be considered and evaluated for LSCC.

Acknowledgements

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