Original Article
Decreased SARI expression predicts poor prognosis of Chinese patients with non-small cell lung cancer

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Abstract: SARI is associated with the risk for several cancers, and loss of SARI expression is frequently found in aggressive and metastatic cancer. Limited evidence shows that SARI is a tumor suppressor gene, but the role of SARI in non-small cell lung cancer (NSCLC) has not been previously reported. This study was to investigate the SARI expression profile in surgically resected lung cancer tissues of Chinese patients by immunohistochemistry and evaluate the relationship between SARI expression and prognosis of lung cancer patients. Furthermore, SARI gene was transfected into lung cancer cells (A549), and the growth curve and cell healing of lung cancer cells were determined, aiming to investigate the influence of SARI on the growth and migration of lung cancer cells in vitro. Results showed that 103 of 195 (52.82%) tissues were positive for SARI. When compared with normal tissues, SARI expression significantly reduced in 50.26% of NSCLC tissues. Patients with negative or reduced SARI expression were more likely to have advanced lung cancer and lymph node metastasis. In squamous carcinoma and adenocarcinoma patients, the SARI expression had no relation with the survival time; However in one-on-one analysis SARI expression in tumor cells and adjacent tissues, patients which tumor cells SARI express reduced than adjacent tissues, survival time was significantly shorter than those without reduction in SARI expression (Log Rank test, \( p = 0.001 \)). After transfection by SARI gene, the proliferation and migration of A549 cells were obviously inhibited \( (p < 0.001) \). These results demonstrate that decreased SARI expression may predict a poor prognosis in NSCLC patients, and SARI may serve as a prognostic biomarker and potential therapeutic target for lung cancer.

Keywords: SARI, non-small cell lung cancer, prognosis, surgically resected cancer

Introduction

Lung cancer is one of the malignant tumors with the highest incidence and mortality [1], and it has been the most common cancer worldwide for several decades. The prognosis of non-small cell lung cancer (NSCLC) is currently unacceptable because the 5-year survival rate is only around 50%, even in patients who undergo radical surgical resection [2]. Therefore, prevention and treatment of lung cancer are a focus of intensive current research.

SARI is a leucine zipper as the basic structure of interferon (IFN) can induce early reaction protein, and belongs to the AP-1 family of transcription factors [3]. However, SARI is different from the traditional proteins in AP-1 family: traditional proteins including JUN (i.e., AP-1), FOS, MAF and so on, often form dimmers accompanied by activation of transcription factor (ATF); this kind of proteins have the basic leucine zipper structure domain (bZIP) and transcription activation area (AD); SARI protein has no AD structure although it contains bZIP structure domain [4-6]. SARI is widely expressed in a variety of normal tissues/cells, but its expression reduces in most tumors [3]. To date, few studies have been conducted to investigate SARI and the signal pathways related to SARI are still


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Unclear. Studies have shown that a variety of normal cell lines have stable SARI mRNA expression. The SARI mRNA expression is the highest in the pancreas and spleen, moderate in the colon, heart, kidney, liver, lung and prostate tissues, and low in the placenta and stomach. However, in their corresponding cancer cells, the SARI mRNA expression is undetectable or declined. This suggests that SARI may inhibit the cancer cell growth. This study aimed to detect the SARI protein expression in surgically resected NSCLC tissues of Chinese patients by immunohistochemistry and evaluate the relationship between SARI expression and prognosis of NSCLC patients with.

Patients and methods

Patients and tissue microarray (TMA)

While 45 paired NSCLC and adjacent normal tissues were collected from 45 patients from January to December in 2010, of whom 20 were diagnosed with lung adenocarcinoma and 25 with lung squamous carcinoma. These patients received surgical intervention and cancers were staged according to the International System of Staging for Lung Cancer (6th edition) [7]. Tissues were formalin-fixed, paraffin-embedded (FFPE) sectioned for observation. The demographics and clinicopathological characteristics (age, gender, stage, tumor grade, lymph node metastasis) at baseline were collected after reviewing medical record. The overall survival was determined from the date of surgery for lung cancer to the time of death. Censored data were defined at the time...
of the last follow up when the date of death was unclear, patients were alive or patients had recurrence. This study was approved by the Ethics Committee of Xinqiao Hospital.

The tissue microarray (diameter, 1.5 mm; depth, 4 μm) was prepared by Outdo Biotech (Shanghai, China) using standard techniques [8]. Additional TMAs were purchased from Shanghai Outdo Biotech.

Cells and cell culture

Lung cancer cell line (A549 cells) was obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (50 μg/ml each of penicillin, streptomycin and gentamicin) at 37°C in a humidified environment with 5% CO₂.

Immunohistochemistry

All tissue sections were routinely deparaffinized, rehydrated, and prepared for immunohistochemistry. Sodium citrate buffer (pH 6) was used as an antigen retrieval solution. Sections were blocked with 5% BSA, and incubated with 1:50 polyclonal anti-SARI antibody (SANTA CRUZ BIOTECHNOLOGY INC. USA) overnight and then with secondary antibodies and DAB reagent (Boshida, Wuhan, China). After dehydration, visualization was done with 3.3-diaminobenzidine (Gene Tech, Shanghai, China). For SARI is mainly expressed in the nucleus, we did not counterstain the nucleus with hematoxylin.

Scoring: The SARI expression was scored by evaluating the staining intensity of positive cells. Positive cells had brown granules, and scoring was done as follows: negative (-), no staining; weak positive (+), positive cells was <50% and staining intensity was low; strong positive (+ +), positive cells were more than 50% or staining intensity was high. The kit had provided positive controls, and TBS was used in the negative control group.

Construction and infection of SARI expression adenovirus vector

T-293 cells (Molecular Genetic Research Third Military Medical University) were maintained, and total RNA was extracted and underwent

Figure 1. Immunohistochemistry analysis. A: SCC SARI (+); B: ADC SARI (+); C: Adjacent Tissue SARI (+); D: SCC SARI (-); E: ADC SARI (-); F: Adjacent Tissue SARI (-).
reverse transcription into cDNA which was used for PCR amplification of bATF gene. The primers were as follows: bATF: 5'-GCCTAAGCC-ATGCACCTCTGT-3', (forward) bATF: 5'-GCTCCG-AAGACCAGGTTAGAAGT-3' (reverse), and the expected size was 849 bp. By using common agarose gel DNA recovery kit (Beijing day root company) recovery of DNA fragments, PCR product through the T4 DNA ligase connection PTA2 carrier, transformation competent Escherichia coli DH5α, with ampicillin LB medium plate screening, incubation overnight, pick take positive clone colony, shake bacteria after alkali cracking method extracted plasmid, after the sequencing results right plasmid named PTA2-BATF; then BATF target gene connection adenovirus skeleton carrier Adtrack, get Adtrack-BATF recombinant plasmid, NotI and HindIII were used to digest cells aiming to confirm whether transfection was successful.
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Results

Clinicopathologic characteristics

A total of 195 NSCLC patients were recruited into this study. The mean age was 62.61 years (range: 37-84 years) and 75.90% of patients were males. All patients received surgical intervention and pathological examination. Squamous cell carcinoma (SCC) was found in 100 patients (51.28%) and adenocarcinoma (ADC) in 95 (48.72%). In addition, 84 (43.08%) patients had lymph node metastasis and 111 (56.92%) had no lymph node metastasis. Moreover, 159 patients were randomly selected to receive follow up. The median duration of follow up was 37.99 months (range: 6-95 months) in survived patients, and a total of 73 (45.91%) patients died during the study. The median survival time was 21.38 months (range: 0-62 months).

Association of SARI expression with lung cancer pathology and clinical stage

Results showed SARI is mainly expressed in the nuclei (Table 1, Figure 1). About 52.82% of cancer cells expressed SARI and almost 88.72% of normal cells in the adjacent tissues expressed SARI. In addition, in 98 cases, SARI expression in the cancer was lower than that in adjacent tissues. There were no relationships of SARI expression in NSCLC with the types of lung cancer, age and sex, but correlation was found with the pathological classification and clinical stage of lung cancer: the higher the differentiation, the higher the rate of SARI expression was (I vs. II, \(p = 0.006\); I vs. III \(p = 0.003\)); the higher rate of the SARI expression and the more advanced the lung cancer, SARI positive expression rate is lower (I vs. II, \(p = 0.003\); I vs. III \(p = 0.001\); II vs. III \(p = 0.003\)); the SARI expression rate in patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis (\(p = 0.001\)).

Association between SARI expression and patients’ survival

The estimated survival time was calculated with Kaplan-Meier method. In squamous carcinoma patients and adenocarcinoma patients, SARI expression had no significant relationship.
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with survival time. However, in a specific patients whose tumor cells had reduced SARI expression as compared to adjacent tissues, survival time was significantly shorter than that of patients without reduced SARI expression in cancer cells (Log Rank test, p < 0.01; Figures 2 and 3).

Proliferation of A549 cells after SARI gene transfection

The A549 cells were transfected with Adtrack-BATF recombinant plasmid for 48 h and MTT assay was done to detect the proliferation of A549 cells. Results showed that the proliferation of transfected cells was significantly inhibited as compared to normal control group and blank control group (p < 0.05, Figure 4).

3.5 Scratch wound healing of A549 cells after SARI gene transfection: The A549 cells were transfected with Adtrack-BATF recombinant plasmid for 24 h, and Scratch wound healing assay was performed to detect the migration ability of A549 cells. Results showed that the migration ability of SARI transfected cells was obviously suppressed when compared with normal control group and blank control group (p < 0.05, Figure 5).

Discussion

Lung cancer is one of the malignant tumors with the highest incidence and mortality, and it has a poor prognosis despite its diagnosis and therapy has been improved. One of the most promising strategies for lung cancer treatment is targeted therapy in which some key genes or pathways are regulated to inhibit the tumor growth. Therefore, it is imperative to identify specific molecular biomarkers of lung cancer.

SARI, (also known as mda-D-74) is an important gene and the melanoma differentiation associated gene (mda), which can make the human melanoma cells irreversible growth inhibition and terminal differentiation in vitro [9]. To date, a series of mda genes have been identified, including mda-5 [10], mda-7 [11], mda-9 [12] and so on. These genes are closely related to the immunity, tumor suppressor and tumor metabolism [13-15].

SARI is a 274 amino acid protein containing one bZIP domain, and has similarity with BATF and BATF3, which belong to the AP-1/ATF superfamily of transcription factors, suggesting that SARI may be involved in the transcriptional regulation. However, SARI gene has its specific structural place, AP-1 transcription factors family (p65, p50, p52, RelB and Rel) and its members are composed of two polymers possessing transcriptional activity [16]. A lot of AP-1 protein contains bZIP and AD domain. However, three BATF proteins have been found to belong to AP-1 protein family and only contain bZIP domain (no AD domain). After binding to AP-1 transcription factor, BATF can inhibit AP-1 tran-
scription activity. SARI is one of above three BATF protein, and can form dimmers in the JUN subunit of AP-1. Through the basic leucine zipper domain, the AP-1 transcription factor’s dimer subunit combination inhibits the AP-1 transcription activity [17]. Recently, Su et al [3] found that over-expression of SARI induced profound growth inhibition and apoptosis in malignant glioma, melanoma and prostate cancer cell lines with no effect on the survival of corresponding normal cells. However, no studies have been conducted to investigate the role of SARI in lung cancer.

In the present study, the SARI expression was detected in cancer tissues and adjacent normal tissues of NSCLC patients. Results showed SARI protein was mainly expressed in the nucleus, and normal tissues had moderate SARI expression. In the cancer tissues (squamous carcinoma and adenocarcinoma), the SARI expression significantly reduced; in patients with advanced NSCLC, the SARI expression was significantly lower than that in patients with lung cancer at early stage (I vs. II, \( p = 0.003 \); I vs. III \( p = 0.001 \); II vs. III \( p = 0.003 \)). In addition, the SARI expression in patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis \( ( p = 0.001 ) \). In addition, the SARI expression and survival time were compared between patients with squamous carcinoma and adenocarcinoma. Results indicated that the SARI expression in cancer tissues had no relation with survival time of patients in both groups. However, in a specific patient, the survival time in patients with reduced SARI expression as compared to adjacent normal tissues was significantly shorter than that in patients without reduced SARI expression \( ( \text{Log Rank test, } p < 0.01 ) \). Therefore, lower SARI expression predicts a poor prognosis of the lung cancer patients which was consistent with the results in the study of Ma, et al [18] on liver cancer.

One of characteristics of cancer cell growth is out of control and abnormal proliferation [3]. In order to further confirm the relationship between SARI expression and lung cancer cell growth and migration, SARI gene was transfected into A549 cells, which were negative for SARI, and the detection of cell growth and scratch wound healing assay were performed. Results showed that the proliferation and migration of A549 cells were significantly inhibited \( ( p < 0.05 ) \) after transfection of by SARI gene. This indicates that the SARI expression can inhibit the proliferation and migration of lung cancer cells, which was consistent with the fact that patients with lung cancer positive for SARI had low grade cancer, were less likely to develop lymph node metastasis and had longer survival time. Thus, we speculated that SARI as an AP-1 inhibitory protein can selectively inhibit cancer growth via the interaction with c-Jun, thus indirectly interfering with the function of transcription factor AP-1 complex, which finally results in the inhibited proliferation and survival of cancer cells [19].

Taken together, our findings showed that decreased SARI expression in Chinese patients with NSCLC had a poor prognosis, and over-expression of SARI in A549 cells by transfection could inhibit the growth and migration of these cells. Thus, we postulate that SARI may serve as a marker for the diagnosis and prognosis of NSCLC and as a target for future treatment of NSCLC.

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Disclosure of conflict of interest

None.

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