Aberrant Expression of SCARA5 in Lung Adenocarcinoma and its Clinical Significance

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Research

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Abstract

Background

Lung adenocarcinoma, the main subtype of non-small cell lung cancer, leading one of the most aggressive and fatal causes of malignant deaths around the world. Scavenger receptor class A member 5 (SCARA5), a newly discovered tumor suppressor gene, belonged to the SR family. The present study's object was to explore the clinical impacts of SCARA5 in lung adenocarcinoma treatment.

Methods

SCARA5 expressions in 120 paired lung adenocarcinoma patients' tissues and cell lines were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC). CCK-8, EdU, flow cytometry, and western blot assays further demonstrated the significance of SCARA5 on A549 cell growth. Then, the relationships between the expression level of SCARA5 and pathological factors were analyzed. Finally, the receiver operating characteristic (ROC) curve and overall survival analysis was carried out to verify the diagnostic and prognostic significance of SCARA5 in lung adenocarcinoma.

Results

SCARA5 was prominently decreased in lung adenocarcinoma cells and tissues compared with human bronchial epithelial cells and para-carcinoma non-tumor tissues. Meanwhile, SCARA5 expression was strongly correlated with smoking (P = 0.0011), TNM stage (P < 0.0001) and lymph node metastasis (P = 0.0005). Furthermore, SCARA5 may be a crucial biomarker for lung adenocarcinoma diagnosis with an area under the curve (AUC) of 0.9102 while SCARA5 could significantly reduce overall survival (OS; P = 0.0006). In vitro experiments, we found that SCARA5 overexpressing could significantly hinder A549 cell proliferation but facilitate apoptosis through the AKT signaling pathway.

Conclusions

SCARA5 might be an important diagnostic and prognostic biomarker for patients with lung adenocarcinoma.

Background

Lung adenocarcinoma was more common in Asian women patients with a smoking history (1,2). The incidence of lung adenocarcinoma was on a rise year by year, accounting for over 40% of all lung cancer, with the overall survival rate remaining low (3). Lung adenocarcinoma patients usually had no obvious particular symptoms at an early stage, generally consisted of fever, hemoptysis, chest pain, shortness of breath, and other respiratory symptoms or asymptomatic (4). Hence, it was easy to be ignored. In recent years, lung adenocarcinoma became common among women, non-smokers, and young adults (5). Most lung cancers were frequently diagnosed at an advanced stage due to the absent symptoms leading to
poor prognosis (6,7). Therefore, it is urging to investigate or reveal diagnostic and prognostic molecular biomarkers for lung adenocarcinoma.

Scavenger receptors (SR) represented a group of heterogeneous molecules located on the surface of phagocytes (8), which could identify oxidized or acetylated modified low-density lipoprotein (LDL). SR was first discovered in 1979 by studying the binding sites on macrophages that ingested and degraded acetylated LDL (9). Later, it was classified as class A scavenger receptor (SR-A) (10). Gradually, several SR-As were subsequently discovered, and these SRs formed an SR family. Scavenger receptor class A member 5 (SCARA5) was a member of the SR family (11), which was ubiquitous in the presence of testis, bladder, trachea, adrenal gland, skin, lung, ovary, thymus, and small intestine (12,13). Studies reported that the essential functions of SCARA5 included the role of organism defense (14). A variety of findings also verified that SCARA5 exerted a pivotal role in tumor growth and angiogenesis inhibition (inhibition of tumorigenesis, colony formation, and cell infiltration) and iron transport (15,16). More importantly, the inhibitory effect of SCARA on the tumor was strong, whose tumor inhibitory effect was 69.4%, the tumor proliferation index was reduced by 23.3%, and the apoptosis index was 47.3% angiogenesis was significantly reduced (17). Previous studies reported that SCARA5 was dysregulated in various cancers, including colorectal cancer (18), breast cancer (19), oral squamous cell carcinoma (20), hepatocellular carcinoma (21), and lung cancer (17,22). However, the clinical impacts of SCARA5 in lung adenocarcinoma has not been elucidated yet.

The purpose of this study was to reveal the eccentric expression and roles of SCARA5 lung adenocarcinoma.

Methods

Patients

120 paired tumor tissues and para-carcinoma non-tumor tissues were obtained from patients with lung adenocarcinoma who had undergone surgical resection without receiving any preoperative adjuvant therapy in the Second Affiliated Hospital, Zhejiang University School of Medicine from May 2013 to April 2015. Written informed consent was obtained from each patient, and the Ethics Committee approved the present study of the Second Affiliated Hospital, Zhejiang University School of Medicine. The pathological parameters of all patients were presented in Table 1.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

The RT-qPCR analysis was used to analyze the abnormal expressions of SCARA5 in tissues and cells, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) functioned as the internal control. Briefly, total RNA was isolated and extracted from tissues or cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) under the manufacturer's instructions. Then, RNA was reverse transcribed into cDNA with the ExScript RT-PCR kit (Takara, Dalian, China). The expression of SCARA5 was detected by SYBR Green PCR Master Mix kit (Applied Biosystems Life Technologies, Foster City, CA, USA) following the
All the amplification and detections were performed in the CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA), and the thermo-cycling profiles were described as follows: 95°C for 10 seconds, 40 cycles of 95°C for 5 seconds, and 62°C for 30 seconds. Statistical analysis was calculated using the 2^-△△CT method.

**Immunohistochemistry (IHC)**

Tissues gathered from 120 patients with lung adenocarcinoma were fixed and embedded in paraffin. Immunohistochemistry was carried out using a rabbit anti-SCARA5 antibody (1:100; ab6118894; Abcam, Shanghai, China). Briefly, the 4 μm tissue sections were deparaffinized and rehydrated. Then, 3% H₂O₂ and 4% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) were applied to block endogenous peroxidase and nonspecific endogenous antigens, respectively. Afterward, the treated sections were incubated with an anti-SCARA5 antibody overnight at 4°C. After washing, the sections were then incubated with IgG H&L secondary antibody (1:1000; ab150077; Abcam, Shanghai, China) at room temperature for 40 min. Last, sections were visualized with DAB (ml016974; Mlbio, Shanghai, China) followed by counterstaining with hematoxylin (ml015306, Mlbio, Shanghai, China) and analyzed with light microscopy.

**Cell culture and transfection**

Human normal bronchial epithelial cell line BEAS-2B and human lung adenocarcinoma cell line A549 were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States). Following the protocol, these two cell lines were cultured in PRMI-1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (Solarbio Life Science, Beijing, China) at a humidified atmosphere with 5% CO₂ at 37°C.

A549 cells were plated in a 24-well plate and transfected with pcDNA3.1 or pcDNA3.1-SCARA5 plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, United States) as per the manufacturer’s protocol. pcDNA3.1 and pcDNA3.1-SCARA5 plasmids were designed and synthesized by GenePharma (Shanghai, China) under the manufacturer’s instructions.

**Cell viability measured by CCK-8 assay**

A549 cells were plated in a 96-well plate with a density of 1×10⁴ cells/well. After the cells reached 70% confluency, cells were transfected with pcDNA3.1 or pcDNA3.1-SCARA5. Then, at 0 hour, 12 hours, 24 hours, and 48 hours, 10 μL CCK-8 reagent was added to each plate and incubated for another 30 minutes at room temperature. Finally, the cell viability was analyzed by measuring the absorbance at 450 nm using a Microplate Reader.

**Cell proliferation rated evaluated by EdU staining**
The transfected cells were inoculated in a 96-well plate with a density of $4 \times 10^3$ cells/well. Then, 100 µL 50 µM EdU reagent was added to each well and cultured for 2 hours. After washed by PBS twice, 50 µL PBS containing 4% paraformaldehyde solution was added to each well and cultured for 30 minutes at room temperature. Subsequently, 100 µL Apollo® 567 Imaging Kit (Riobio) and Hoechst 33342 were added and cultured at 37°C for 30 minutes. Afterward, 100 µL PBS containing 0.5% Triton X-100 was added and cultured for another 5 minutes at room temperature. Finally, all images were captured with fluorescence microscopy.

**Cell apoptosis detected by flow cytometry analysis**

After transfection, cells were cultured in a 6-well plate at 37°C in a humidified atmosphere. At 48 hours post-incubation, cells were stained with Annexin V/PI using an apoptosis kit. Finally, the cell apoptosis rate was analyzed using flow cytometry following the instructions.

**Apoptosis-related proteins determined by western blot analysis**

At 24 hours post-transfection, total proteins were isolated from cells by RIPA buffer and quantified using a Protein BCA Assay Kit following the manufacturer’s protocol. Then, proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. Afterward, the membranes were blocked with 5% non-fat milk at 37°C for 2 hours and incubated with primary antibodies (anti-AKT, ab38449, 1:1000, Abcam, Shanghai, China; anti-p-AKT, ab38449, 1:1000, Abcam, Shanghai, China; anti-Bcl-2, ab32124, 1:1000, Abcam, Shanghai, China; anti-Bax, ab32503, 1:1000, Abcam, Shanghai, China; and anti-GAPDH, AB9485, 1:2500, Abcam, Shanghai, China) at 4°C overnight. After washing with TBST three times, the membranes were then probed with secondary antibodies (goat-anti-rabbit IgG H&L, ab7090, 1:1000, Abcam, Shanghai, China) at 37°C for 1 hour. Finally, an ECL solution was added to detect protein bands, and ImageJ software was used to quantify the results.

**Statistical analysis**

All analyses were performed using SPSS 17.0 (Chicago, IL, USA) to analyze the data. The expression level of SCARA5 was presented as a mean ± standard deviation via the student's t-test. The $\chi^2$ test was used to determine the correlation of SCARA5 expression and pathological factors in lung adenocarcinoma. The ROC curve defined by the log-rank test and the overall survival by Kaplan-Meier analysis was applied to determine the diagnostic and prognostic value of SCARA5 in lung adenocarcinoma, respectively. A P value < 0.05 was considered to be statistically significant.

**Results**

The expression of SCARA5 was downregulated in lung adenocarcinoma tumor tissues compared with para-carcinoma tissues.
As shown in Fig. 1A, the mRNA expression level of SCARA5 was prominently decreased in lung adenocarcinoma tumor tissues compared with para-carcinoma non-tumor tissues (P<0.001). Consistent with the mRNA result, the protein measured by IHC in Fig. 1B presented a similar trend.

**Relationship of SCARA5 expression with pathological factors in lung adenocarcinoma**

As shown in Table 1, high expression of SCARA5 presented a strong correlation with smoking (P = 0.0011), TNM stage (P < 0.0001) and lymph node metastasis (P = 0.0005). Whereas, there were no significant differences in gender, age, and tumor size between the high and low expression of SCARA5 groups.

**SCARA5 was a diagnostic and prognostic biomarker in lung adenocarcinoma**

As shown in Fig. 2A, the AUC value was 0.9102 (95% CI = 0.8681~0.9523), suggesting that SCARA5 exerted its remarkable function concerning lung adenocarcinoma diagnosis. Meanwhile, Fig. 2B further demonstrated that the overall survival determined by Kaplan-Meier analysis of lung adenocarcinoma patients with low SCARA5 expression was lower than those with high expression.

**SCARA5 was significantly downregulated in lung adenocarcinoma cell compared with normal bronchial epithelial cell**

Through RT-qPCR analysis, we detected the eccentric levels of SCARA5 in lung adenocarcinoma cells. As shown in Fig. 3A, the SCARA5 level was remarkably decreased in A549 lung adenocarcinoma cell compared with normal bronchial epithelial BEAS-2B cell (P<0.01). Furthermore, the transfection efficiency confirmed that SCARA5 levels were prominently increased after transfection with pcDNA3.1-SCARA5, suggesting the transfection was successful (Fig. 3B; P<0.01).

**SCARA5 overexpressing inhibited A549 cell proliferation but promote cell apoptosis via AKT signaling pathway**

After transfection, we further detected the effects of SCARA5 on A549 cell proliferation and apoptosis. Results in Fig. 4 and Fig. 5 showed that SCARA5 overexpressing markedly suppressed A549 cell proliferation compared with the pcDNA3.1 group (P<0.01); however, flow cytometry results in Fig. 6 verified that upregulation of SCARA5 could promote A549 cell apoptosis (P<0.01). As shown in Fig. 7, compared with the pcDNA3.1 group, SCARA5 overexpressing could reduce p-AKT and Bax levels but increase Bcl-2.

**Discussion**

Recently, studies concerning cancer biomarkers have been massively reported for its clinical role in the early diagnosis and prognosis of various cancers (23-25), including lung adenocarcinoma (26,27). As known, the majority of lung adenocarcinoma cancer-related death frequently occurred at an early stage.
Contributions and development of specific biomarkers for lung adenocarcinoma for early diagnosis and prognosis might improve overall survival and clinical situations.

In the present study, the mRNA and protein expression of SCARA5 was downregulated in lung adenocarcinoma tumor tissues compared with para-carcinoma non-tumor tissues, consistent with a previous study (17). Meanwhile, SCARA5 expression was strongly correlated with smoking, TNM stage, and lymph node metastasis. Our findings agreed with the earlier studies on the roles of SCARA5 in hepatocellular carcinoma and breast cancer (19,28). Furthermore, we investigated the diagnostic and prognostic value of SCARA5 in lung adenocarcinoma. As demonstrated, the ROC curve presented an AUC value of and the Kaplan-Meier analysis revealed that patients with low SCARA5 expression’s overall survival were lower than those with higher expression. Thus, the downregulation of SCARA5 in patients may be associated with poor prognosis, and SCARA5 may exert its function as a crucial biomarker concerning lung adenocarcinoma early diagnosis and prognosis.

Consistent with our findings, the clinical significance of SCARA5 was verified in various cancers before. For instance, Liu et al. (18) proposed that SCARA5 was downregulated in colorectal cancer tissues, and low SCARA5 expression was correlated with unfavorable prognosis in colorectal cancer. Yan et al. (17) confirmed that SCARA5 was frequently decreased in various cancer, functioning as potential antimetastatic agents for cancer treatment. In our study, we found that SCARA5 was downregulated in A549 lung adenocarcinoma compared with BEAS-2B normal bronchial epithelial cells; meanwhile, SCARA5 overexpressing could inhibit lung adenocarcinoma cell proliferation but promote apoptosis through the AKT signaling pathway.

The present study identified the clinical value of SCARA5 in predicting lung adenocarcinoma diagnosis and prognosis, suggesting that it may be an essential biomarker for therapeutics of lung adenocarcinoma.

**Abbreviations**

SCARA5: Scavenger receptor class A member 5

RT-qPCR: real-time quantitative polymerase chain reaction

IHC: immunohistochemistry

ROC: receiver operating characteristic

AUC: area under the curve

LDL: low-density lipoprotein

GAPDH: glyceraldehyde 3-phosphate dehydrogenase
Declarations

Ethics approval and consent to participate

Written informed consent was obtained from each patient, and the Ethics Committee approved the present study of the Second Affiliated Hospital, Zhejiang University School of Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors’ contributions

YXG, GXF, FAX, HY, XJP, WN and WQC collected samples, analyzed and interpreted the patients data. ZW, HHL, OYJJ and FL contributed to the experimental design, manuscript drafting and revising. All authors read and approved the final manuscript.

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Declaration of interest: None.

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**Tables**

**Table 1 Relationships between SCARA5 expression and pathological factors in 120 patients with lung adenocarcinoma.**
| Characteristics          | Cases | SCARA5 expression | P-value |
|-------------------------|-------|-------------------|---------|
|                         |       | Low (n=76)       | High (n=44) |
| Gender                  |       |                  |          |
| Male                    | 36    | 19               | 17       | 0.1366 |
| Female                  | 84    | 57               | 28       |        |
| Age (years)             |       |                  |          |
| ≤ 50                    | 47    | 32               | 15       | 0.3861 |
| > 50                    | 73    | 44               | 29       |        |
| Size (cm)               |       |                  |          |
| ≤ 2.0                   | 51    | 33               | 18       | 0.7885 |
| > 2.0                   | 69    | 43               | 26       |        |
| Smoking                 |       |                  |          |
| Yes                     | 86    | 63               | 23       | 0.0011 |
| No                      | 34    | 13               | 21       |        |
| TNM stage               |       |                  |          |
| T1/T2                   | 41    | 11               | 30       | < 0.0001 |
| T3/T4                   | 79    | 65               | 14       |        |
| Lymph node metastasis   |       |                  |          |
| No                      | 88    | 63               | 25       | 0.0005 |
| Yes                     | 32    | 13               | 21       |