Over-expression of Orai1 mediates cell proliferation and associates with poor prognosis in human non-small cell lung carcinoma

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Abstract: Orai1 and STIM1 mediate calcium release-activated calcium current (CRAC) which is the best characterized store-operated calcium current involving in a wide range of cell progresses, such as cell proliferation, metastasis, apoptosis. Orai1 has been studied as a carcinogenic biomarker in some cancers such as esophageal cancer. However, its function and clinical significance in non-small cell lung cancer (NSCLC) have not been well studied. The present study was aimed at discussing the relationship between Orai1 and lung cancer malignant behavior with its clinical significance. We used quantitative real-time-PCR and Western blot to detect the expression of Orai1 in NSCLC cell lines and fresh cancer tissues. Immunohistochemistry were performed to test the location and expression of Orai1 in paraffin sections. We found that Orai1 was markedly overexpressed in both NSCLC cell lines and fresh cancer tissues. Immunohistochemistry data also revealed that overexpression of Orai1 was present in 42.4% of NSCLC tissues, compared with the corresponding adjacent nontumorous tissues. Furthermore, NSCLC patients with high Orai1 expression survived shorter than those with low Orai1 expression. In addition, when knockdown Orai1 by RNAi technic, we found the PI3k/AKT/ERK pathway was inhibited which may indicated that Orai1 could influence cell proliferation. Taken together, our study demonstrated that Orai1 was remarkably overexpressed in NSCLC and could be served as a potential prognostic marker for patients with this deadly disease.

Keywords: Orai1, NSCLC, proliferation, ERK, AKT

Introduction

The store-operated calcium entry (SOCE) channel is the major Ca2+ entry pathway in non-excitable cells [1, 2]. SOCE is a ubiquitous cellular mechanism linking the calcium depletion of the endoplasmic reticulum (ER) to the activation of plasma membrane (PM) Ca2+-permeable channels [3]. The essential components of SOCE are Orai1 and STIM1 proteins [1, 2]. Orai1 is a pore-forming component of SOCE while STIM1 serves as a sensor of the luminal Ca2+ concentration with its EF-hand Ca2+-binding domain, a canonical helix-loop-helix Ca2+-binding motif [4, 5].

Orai1 is a four-transmembrane domain plasma membrane Ca2+-permeable channel localized in PM. STIM 1 is a single transmembrane protein mainly localized in EM. The N-terminal of STIM 1 is located in the lumen of ER and functions as a Ca2+ sensor. The C-terminal of STIM 1 is in the cytosol and interacts with Orai1 to activate SOCE upon store depletion [6]. Orai1 and STIM1 mediate calcium release-activated calcium current (CRAC) which is the best characterized store-operated calcium current involving in a wide range of cell progresses, such as cell proliferation, metastasis, apoptosis. In normal cells, the expression of Orai1 and STIM1 was also detected and the expression level was varied in different cells. Chen, Y.F et al. showed that the Orai1/STIM1 expression level is higher in cervical cancer tissues than adjacent non-neoplastic tissues. Many studies have demonstrated that the disorder of Orai1-and STIM1-SOCE channel in normal tissues can result in T cell activation defect, congenital thrombocytopenia and other diseases [7]. However, it has not any relative reports on Orai1 expression status in NSCLC.
Lung cancer is the 1th leading cause of human cancer death worldwide [8]. Non-small cell lung cancer (NSCLC) is the key component of lung cancer and accounts for more than 80%. Although many therapeutic strategies take an advantage in treatment of NSCLC, most patients suffering from NSCLC showed a poor 5-year survival rate [9]. Identification of biomarkers for early detection, prognostic stratification, and novel therapeutic interventions are therefore urgently needed for effective management of NSCLC.

The present study was undertaken to reveal the expression profile of Orai1 in tumor tissues from patients with NSCLC and more importantly to explore the clinical significance of Orai1 in lung cancer. We performed functional analysis on Orai1 in NSCLC with in vitro approaches. Our attempt is to identify any abnormity in SOCE to be used as diagnostic and/or prognostic biomarker and to provide insights to mechanistic understanding on how such abnormity in SOCE pathway regulates tumor progression.

**Materials and methods**

**Chemicals and reagents**

MTT, DMSO were products of Sigma Chemical Co. Lapatinib was obtained from AstraZeneca Pharmaceuticals. RPMI 1640 and DMEM were products of Gibco BRL. Monoclonal antibodies against Orai1, GAPDH, ERK1/2, p-ERK, AKT and p-AKT were products of Santa Cruz Biotechnology, Inc. Other routine laboratory reagents were obtained from commercial sources of analytical grade.

**Patients and tissue specimens**

All protocols concerning human subjects were approved by the Regional Ethical Committee of Nanchang University, and all investigations with human subjects were conducted after informed consent was obtained. Primary tumors and their neighboring non-tumorous tissues were obtained from 22 patients with NSCLC, each of whom underwent surgical resection without preoperative systemic chemotherapy at the First Affiliated Hospital of Nanchang University. The specimens were collected immediately after surgical removal and fixed with 10% formalin followed by paraffin-embedding. The median follow-up period was 25 months (range: 1-96 months). Tumor histology confirmed that all specimens were ESCC and that 53 of 82 (64.6%) tumors originated in the thorax. For Western blot and quantitative RT-PCR expression studies, 34 pairs of fresh samples were frozen in liquid nitrogen immediately after surgical removal and maintained at -80°C until use.

**Plasmids and transfection**

Multiple short hairpin RNA (shRNA) probes targeting the human orai1 gene and a probe containing a scrambled sequence (control) were designed and constructed into a pU6-mRFP expression vector as described previously [26]. H-1299 cells were transfected with Orai1 siRNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions with the following target sequences: Orai1 shRNA #1: 5'-TCGGCCTGATCTTTATC-3', and Orai1 shRNA #2: 5'-CCAGCATTGTGTGTACA-3' [10]. They were verified by Western blot analysis to be effective in silencing the Orai1 gene.

**Immunohistochemistry**

Immunohistochemistry (IHC) analysis for Orai1 was performed using a standard two-step method. TMA sections were baked overnight at 37°C, and then deparaffinized and rehydrated. Slides were boiled in Ethylene Diamine Tetraacetic Acid (EDTA; 1 mmol/L; pH 8.0) in a pressure cooker for antigen retrieval. Subsequently, slides were incubated overnight at 4°C with Orai1 antibody (1:500 dilution). After rinsed with PBS, the slides were incubated with a secondary antibody and stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB). Finally, the slides were counterstained with Mayer’s hematoxylin. Slides immunoreacted with PBS were used as the negative controls. Stained cell proportions were scored as follows: 0 (less than 5% stained cell); 1 (6-24% positively stained cells); 2 (25-49% positively stained cells); 3 (50-74% positively stained cells); 4 (75%-100% positively stained cells). Staining intensity was graded according to the following standard: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown) and 3 (strong staining = brown). The product of [positively stained cell proportion x stained intensity] served as the receptor score. The median value of IHC scores was 4;
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Figure 1. Expression of Orai1 in NSCLC tissue samples detected by qRT-PCR and western blot. A&B. Protein levels of Orai1 in NSCLC tissue samples by western blot. A. Representative images of Orai1 expression were presented. B. The ratio of Orai1/GAPDH was calculated by student paired t test. C. The mRNA levels of Orai1 in 22 pairs of NSCLC and corresponding adjacent lung tissues were examined. Relative Orai1 mRNA expression level was presented. D. Significance of alteration of Orai1 mRNA was revealed by Wilcoxon matched paired test.

Figure 2. Relationship between Orai1 expression and NSCLC prognosis. Orai1 protein level showed prognostic role in Overall survival (A), Disease-free survival (B), as indicated by Kaplan-Meier analysis. Statistical significance was assessed with the log-rank test (A, \( P = 0.002 \); B, \( P = 0.012 \); n = 56).

cell culture

The human non-small cell lung cancer cell line H1299 was cultured in RPMI 1640 with 10% of fetal bovine serum (FBS). The normal lung cell BEAS-2B was cultured in RPMI 1640 with 10% of fetal bovine serum (FBS). All cells were incubated in a humidified atmosphere of 5% \( \text{CO}_2 \) and 95% air at 37°C.

cytotoxicity test

MTT assay was used to assess anti-proliferation activity. Briefly, cells were seeded in 96-well plates and allowed to attach overnight. Briefly,
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A

Patient 1

Patient 2

Patient 3

B

$P < 0.01$
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3-5 × 10^3 cells were incubated in 96-well plates and allowed to attach overnight. To determine the cytotoxicity, the drugs at various concentrations were added to the cells and the cells were incubated at 37°C for 68 h. MTT (5 mg/mL, 20 µL/well) was then added to the cells for 4 h (37°C), and then DMSO (120 µL/well) was added to dissolve the formazan. Finally, optical density was measured at 540 nm with 655 nm as a reference filter by Model 550 Microplate Reader (Bio-Rad, USA). Experiments were performed at least three times.

Preparation of cell lysates and western blot analysis

The cells were harvested and rinsed twice with ice-cold PBS buffer. Cell extracts were collected in cell lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 10 mg/mL leupeptin). Equal amounts of cell lysates from various treatments (30 µg of protein) were resolved by SDS-PAGE and then electrophoretically transferred onto PVDF membranes. After blocked in 5% non-fat milk in TBST buffer (10 mmol/L Tris-HCL, 150 mmol/L NaCl, and 0.1% Tween20, pH 8.0) for 1 h at room temperature, the membranes were incubated with appropriately diluted primary antibodies overnight at 4°C. The membranes were then washed thrice with TBST buffer and incubated with HRP-conjugated secondary antibody at 1:5000 dilutions for 2 h at room temperature. After washed thrice with TBST buffer, the protein-antibody complex were visualized by the enhanced Phototope TM-HRP Detection Kit (Cell Signaling, USA) and exposed to Kodak medical X-ray processor (Carestream Health, USA). GAPDH was used as the loading control.

Reverse transcription-PCR and Q-PCR

The total cellular RNA was isolated by Trizol Reagent RNA extraction kit following manufacturer’s instruction (Molecular Research Center, USA). The first strand cDNA was synthesized by Oligo dT primers with reverse transcriptase (Promega Corp). The reactions were carried out at 94°C for 2 min for initial denaturation, and then at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. After 35 cycles of amplification, additional extensions were carried out at 72°C for 10 min. Products were resolved and examined by 1% agarose gel electrophoresis. Q-PCR was performed by the Bio-Rad CFX96TM Real-Time (Applied Biosystems, USA). The geometric mean of the β-actin was used as an internal control to normalize the variability in expression levels. The PCR reactions were performed at 50°C for 2 min, 95°C for 5 min and 40 cycles at 95°C for 15 s, 60°C for 30 s. Relative quantification of Orai1 was performed using the 2-ΔΔCt method. To ensure reproducibility of the results, all genes were tested in triplicate in three independent experiments. Primers were designed as follows: Orai1, forward: 5'-GACTGGATCGGCCAGAGTTAC-3' and reverse: 5'-GTCCGGCTTGGAGGCTTTAG-3'; β-actin, forward: 5'-CACCATGAAGATCAAGA-
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Figure 4. Orai1 siRNA significantly decreased cell proliferation in NSCLC cells. (A) Orai1 siRNA noticeably silenced the protein expression of Orai1. Scramble and Orai1 siRNA were transfected into H1299 cells for 48 h. Relative Orai1 expressions were detected by western blot. (B) The cell viabilities were determined using MTT assays. The data are calculated as means ± SD of three independent experiments. (C&D) Knockdown Orai1 significantly reduced the p-ERK and p-AKT level. Representative images of p-ERK and p-AKT levels were presented.

TCATTGC-3' and reverse: 5'-GGCCGGACTCATC-GTACTCCTGC-3'.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, IL, USA). The Students't test was used for comparison between groups. The x² test was performed to analyze the correlation between Orai1 expression and clinicopathological parameters. The Kaplan-Meier method (the log-rank test) was used for survival curves. P < 0.05 (two-tailed) was considered statistically significant.

Results

mRNA and protein levels of Orai1 in NSCLC tissues

We detected Orai1 expression in 22 paired NSCLC and the corresponding adjacent non-tumor tissues, using qRT-PCR and western blot. Compared with nontumorous lung tissues, the protein level of Orai1 was obviously upregulated in NSCLC tissues (Figure 1A and 1B). Consistently, in 20 out of 22 (90.9%) cases, Orai1 mRNA was overexpressed in tumor tissues, compared with the adjacent nontumorous tissues (Figure 1C and 1D). Besides, Orai1 was significantly higher expressed in NSCLC paraffin-embedded tissues than non-tumor lung tissues (Figure 3B).

Association of high Orai1 expression in NSCLC with poor survival

The association between Orai1 expression in NSCLC and the survival of selected patients was analyzed with Kaplan-Meier survival analysis. Patients with high Orai1 expression were likely to be with significantly shorter overall survival, disease-free survival and metastasis-free survival, but not recurrence-free survival. Since
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Orai1 expression was significantly corrected to AFP, tumor size and clinical stage, we further determined the relationship between Orai1 expression and the survival of patients subclassified as ‘large tumor’ and ‘Stage (III-VI)’. As showed by Figure 2, patients in subclassified groups with high Orai1 expression survived shorter than those with high Orai1 expression.

Relationship between Orai1 expression level and clinicopathological parameters of NSCLC

IHC was performed to assess the expression of Orai1 in 56 paraffin-embedded NSCLC tissues. The data revealed that Orai1 was mainly expressed in the membrane of cancer cells (Figure 3A-3E). Scattered staining of Orai1 in nuclear was also observed. As indicated by Figure 3F, high Orai1 expression in tumor tissue was found in 36 out of 56 cases. The relationship between Orai1 expression and clinicopathological parameters was further analyzed. NSCLC patients with high Orai1 expression had a higher tendency to be with advanced clinical stage ($P = 0.034$), high incidence of metastasis ($P = 0.019$) and large tumor size ($P = 0.032$). There was no statistical connections between Orai1 expression and other clinicopathological factors, such as age, gender, tumor differentiation and the incidence of vascular invasion (Table 1). By Kaplan Meier survival analysis, we found the association between Orai1 expression status and the survival time of patients with NSCLC. Patients with higher Orai1 expression were likely to be with significantly shorter overall survival ($P = 0.002$) and disease-free survival ($P = 0.012$).

Knockdown of Orai1 suppressed cell proliferation in NSCLC cells

We next examined whether a decrease in Orai1 could affect NSCLC cell viability. Two Orai1 siRNAs were proven to effectively down-regulated the expression of Orai1 (Figure 4A). The knockdown of Orai1 in H1299 cells led to a decrease of cell viability (Figure 4B), as indicated by MTT assay. In addition, phosphorylated ERK (p44/42) and phosphorylated AKT (T308) were reduced when silenced Orai1 in vitro experiment. Those data indicated that Orai1 knock-out could injury cell viability and the ERK as well as AKT pathways act as a vital role in this process.

Discussion

NSCLC is a heterogeneous cancer with high mortality. Searching for valuable biomarkers for NSCLC diagnosis and prognostic prediction has been attracting an increasing amount of interest. Plenty of proteins, such as EGFR, RET and VEGFR2, have been shown to have clinical significance for predicting NSCLC prognosis [11-13]. Specific targeting of some proteins such as EGFR in cancer cells can prevent cell proliferation, metastasis and promote cell death. Some reports showed that knockdown Orai1 with siRNA can block the Ca2+ entry pathway resulting in cell apoptosis and the inhibition of cell growth [14-16]. The Orai1- mediated Ca2+ signaling is an attractive target for therapeutic strategy. Lots of studies in vivo or in vitro have affirmed that knockdown Orai1 could inhibit cell migration, induce cell apoptosis and suppress cancer cell tumor growth [17-19]. In this study, we aimed to investigate the expression and the prognostic value of Orai1 in NSCLC.

Our data showed that the Orai1 protein level was increased in NSCLC patients and was associated with unfavorable prognosis. Orai1 expression level was inversely correlated with both overall and disease-free survival of NSCLC patients. Increased levels of Orai1 were observed in other human cancers such as esophageal carcinoma, colon cancer [20]. Some reports demonstrated that overexpression of Orai1 was associated with tumor progression and poor prognosis in colon cancer [20]. Yang S et al. showed that Orai1 overexpression in breast cancer was correlated with poor prognosis [5]. Increased expression of Orai1 was observed in malignant glioma [21]. In the present study, our data suggesting Orai1 may be involved in NSCLC progression. The detailed mechanism by which Orai1 is up-regulated in NSCLC requires future investigation.

Compared with that in neighboring non-tumorous lung tissues, the expression of Orai1 in tumors obtained from patients with NSCLC was significantly elevated. High Orai1 expression was strongly associated with either overall survival or disease-free survival. Moreover, inhibition of Orai1-mediated SOCE by Orai1 knockdown damaged the proliferation ability of NSCLC cells.
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Taken together, although the detailed mechanism remains unclear, Orai1 can function as a tumor marker in NSCLC and is of clinical significance in predicting the postsurgical prognosis of patients who suffer from this deadly disease.

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Disclosure of conflict of interest

None.

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