14-3-3 ζ Regulates Immune Response through Stat3 Signaling in Oral Squamous Cell Carcinoma

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Ectopic expression of 14-3-3 ζ has been found in various malignancies, including lung cancer, liver cancer, head and neck squamous cell carcinoma (HNSCC), and so on. However, the effect of 14-3-3 ζ in the regulation of interactions between tumor cells and the immune system has not been previously reported. In this study, we aimed to investigate whether and how 14-3-3 ζ is implicated in tumor inflammation modulation and immune recognition evasion. In oral squamous cell carcinoma (OSCC) cell lines and cancer tissues, we found that 14-3-3 ζ is overexpressed. In OSCC cells, 14-3-3 ζ knockdown resulted in the up-regulated expression of inflammatory cytokines. In contrast, 14-3-3 ζ introduction attenuated cytokine expression in human normal keratinocytes and fibroblasts stimulated with interferon- γ (IFN- γ) and lipopolysaccharide (LPS). Furthermore, supernatants from 14-3-3 ζ knockdown OSCC cells dramatically altered the response of peritoneal macrophages, dendritic cells and tumor-specific T cells. Interestingly, Stat3 was found to directly interact with 14-3-3 ζ and its disruption relieved the inhibition induced by 14-3-3 ζ in tumor inflammation. Taken together, our studies provide evidence that 14-3-3 ζ may regulate tumor inflammation and immune response through Stat3 signaling in OSCC.

INTRODUCTION

Head and neck cancer including oral cancer has become the sixth most common cancer worldwide and a notable concern with increasing incidence in young and middle-aged men (Bettendorf et al., 2004; Parkin et al., 1999). It has been reported that oral squamous cell carcinoma (OSCC) is an important component of the worldwide burden of cancer, which bears a 5-year survival rate of approximately 50% and is poorer than breast cancer or melanoma (Jemal et al., 2011). To explore a promising method of OSCC treatment and prognosis, a better

understanding of the molecular mechanisms behind the development of this disease would be helpful.

14-3-3 is a family of highly conserved proteins that are ubiquitously expressed in various mammalian tissues (Aitken, 1996; Reuther et al., 1996). There are seven isoforms (β , γ , ϵ , η , σ , τ , and ζ) of 14-3-3 encoded in humans (Hashiguchi et al., 2000; Ichimura et al., 1988). Evidence has revealed that dimerized 14-3-3 molecules may exert their function by binding to specific motifs, the most widely accepted of which are RSXpSXP and RXXXpSXP (Lin et al., 2009; Yaffe et al., 1997).

As a pro-oncogenic gene, the expression of 14-3-3 ζ is negatively associated with prognosis in breast cancer patients (Neal et al., 2009). 14-3-3 ζ may directly bind to PI3 kinase, promote Akt activation and enhance cell survival (Neal et al., 2012), as well as inactivate tumor suppressors like p53 and p21 (Danes et al., 2008; Wang et al., 2010). Furthermore, Matta et al. demonstrated that 14-3-3 ζ is overexpressed in pre-malignant stages of oral cancer, even in hyperplasia, and that its high-expression is sustained further downstream in the tumorigenic pathway (Matta et al., 2007). Additionally, 14-3-3 proteins have been involved in the regulation of TLR signaling, whereby the stimulation of RAW cells with LPS led to PKC phosphorylation and its association with 14-3-3 ζ in a MyD88-dependent manner (Faisal et al., 2008). Also, 14-3-3 ζ was reported to have opposite effects on TLR2 and TLR4 signaling (Schuster et al., 2011), whereby 14-3-3 ζ inhibited TLR2-mediated NF- κ B activation but enhanced TLR4-mediated NF- κ B activation. However, it still remains unclear whether and how 14-3-3 ζ is involved in tumor inflammation and immune regulation.

The signal transducer and activator of transcription (STAT) family is reported to contain seven members in mammals (Levy et al., 2002; O'Shea et al., 2013). Stat3, the one mostly studied, is implicated in several biological processes. Stat3 may promote tumor growth through its multifunctional role, which includes implicating in cell metastasis, augmenting tumor angiogenesis, and protecting tumors from innate immune recognition (Silver et al., 2004; Wang et al., 2005; Wei et al., 2003). Notably, the Stat3 signaling can be functionally modulated by its interaction with numerous other proteins including 14-3-3 ζ and communicate with various other signaling cascades like the NF- κ B, AP-1 or PI-3K pathways (Yu et al., 2007).

The purpose of the present study was to investigate the role of 14-3-3 ζ in tumor inflammation and immune response. In the present study, we found that 14-3-3 ζ is overexpressed in OSCC cell lines and cancer tissues. 14-3-3 ζ knockdown led to increasing inflammatory cytokine expression, while 14-3-3 ζ introduction attenuated cytokine production. Moreover, conditioned

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medium from 14-3-3 ζ -disrupted tumor cells triggered increased innate and adaptive immune responses. In addition, 14-3-3 ζ interacts and promotes the nuclear translocation of Stat3 which has been demonstrated to be essential for tumor inflammation.

MATERIALS AND METHODS

Cell culture and tissue collection

The human tongue SCC cell line Cal27 was purchased from American Type Culture Collection (ATCC, USA). Cell line Tca8113 was purchased from the Chinese Academy of Sciences (Peking, China). These two cell lines were routinely cultured in RPMI 1640 medium (Gibco, USA) with 10% fetal calf serum (Gibco, USA) and 1% penicillin/streptomycin at 37°C in an incubator (SANYO, Japan) with 5% CO₂. The medium was changed every 2-3 days and cells were digested using trypsin and were sub-cultured in a new plate when they reached ~80% confluence. Normal human oral keratinocytes were prepared and maintained as previously described (Min et al., 2004). Briefly, keratinocytes were isolated from human gingival tissue specimens obtained from healthy volunteers (20-30 years old) who were undergoing oral surgery. Oral keratinocytes were isolated from separated epithelial tissue by trypsinization, and primary cultures were established in a keratinocyte growth medium containing 0.15 mm calcium and a supplementary growth-factor bullet kit (KGM; Clonetics, USA). Primary normal human gingival fibroblasts were established from the explant cultures of gingival connective tissue, which was excised from a patient undergoing oral surgery. The cells that proliferated outwardly from the explant culture were continuously cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Primary OSCC cells derived from the oral cavity were obtained from surgeries performed at the First Affiliated Hospital of Zhengzhou University and delivered to our laboratory. All adjacent specimens were harvested from morphologically normal appearing tissue located at least 3 cm from the tumor and were used as the normal control tissue for comparisons with the tumor. Normal mucosal cells were harvested from 21 healthy adults and used as a control. Samples were snap-frozen in liquid nitrogen and stored at -80°C before RNA isolation. The protocols were approved by the Committee for Ethical Procedures of Zhengzhou University, and the study was performed in accordance with the Declaration of Helsinki (2008) for humans.

ELISA analysis

The levels of inflammatory cytokines in supernatants were evaluated with a commercially available enzyme-linked immunosorbent assay (ELISA; Yanjin Biotechnology Co., China), according to the manufacturer's instructions. The absorbance was read at 450 nm using a 680XR Microplate reader (Bio-Rad, USA). All of the samples were analyzed in duplicate. The standard curve for protein estimation was made by linear regression analysis.

Adenovirus construction

All recombinant adenoviruses were constructed according to a previous report (He et al., 1998). Briefly, 14-3-3 ζ was amplified and sub-cloned into pAdTrack-CMV, an adenoviral shuttle plasmid, whereas GFP was used as a non-specific control. Then, the recombinant shuttle plasmids pAdTrack-CMV and pAdEasy-1 were homologously recombined in the *Escherichia coli* strain BJ5183. The recombinant plasmids obtained were trans-

ected into 293 cells to generate recombinant adenovirus. The virus was amplified and purified, and titers were determined by p24 ELISA kit (Cell Biolabs, Inc., USA), before being stored at -80°C for subsequent use.

siRNA transfection

Scrambled siRNA and small-interfering RNA (siRNA) targeting 14-3-3 ζ (sc-29583) or Stat3 (sc-29494) were purchased from Santa Cruz Biotechnology (USA). Cells were transfected with scrambled or 14-3-3 ζ /Stat3 siRNA according to the manufacturer's protocol. Briefly, 14-3-3 ζ or Stat3 and scrambled siRNAs (30 pmol) were diluted in 500 μ l DMEM and mixed with 5 μ l Lipofectamine RNAi MAX (Invitrogen, USA). After 15 min of incubation at room temperature, the complexes were added to the cells to a final volume of 3 ml medium. Cells were then harvested at the indicated times for further analysis. The efficiency of 14-3-3 ζ or Stat3 siRNA was confirmed by Western blot analysis of Flag expression.

MTT assay

T cell growth and proliferation was evaluated by MTT assay. The experiments were carried out in 96-well plates according to the manufacturer's protocols (Roche GmbH, Germany). In the MTT test, tetrazolium salts were transformed by active enzymes of the cells into intracellular formazan deposits and cells were incubated for 4 h with the tetrazolium salts. After this incubation time, the purple formazan salts formed became soluble. Absorbance was determined at 490 nm.

Reporter gene assays

OSCC cells were infected with adenovirus-NF- κ B-luciferase adenovirus (at 10⁷ pfu/ml). Then, 24 and 48 h after infection, cells were collected and washed with ice-cold PBS, lysed using 250 μ l Passive Lysis Buffer (Promega, USA), and centrifuged (13,000 rpm for 10 min at 4°C). Assays for luciferase activity were performed according to the manufacturer's protocol (Promega) and measured using a luminometer (Veritas; Symantec) and GloMax software (Promega).

Cell fractionation

Cal27 cells were transfected with scrambled or 14-3-3 ζ siRNA and then harvested at 24 h after the transfection. Cell fraction was performed with a nuclear and cytoplasmic extraction reagents (Invitrogen, USA) according to the manufacturer's instructions.

Flow cytometry

Dendritic cells were incubated with the FITC-conjugated specific antibody (60 min, 4°C) and analyzed in a Becton Dickinson FACScan flow cytometer (USA), as described previously (Kwak et al., 2000). At least 100,000 viable cells were analyzed per condition. Data were analyzed using CELLQUEST software (Becton Dickinson).

Immunocytochemistry

Cal27 cells (5 $\times$ 10⁴) were plated on 13-mm glass coverslips coated with poly-L-lysine (Sigma, USA) in 0.5ml of RPMI 1640 medium (Gibco, USA) with 10% fetal calf serum per well of a 24-well plate. After overnight incubation, 100 ng of pSVL-STAT3-YFP plasmid DNA (Addgene, USA) was co-transfected with 14-3-3 ζ siRNA. After 24 h, cells were transferred to serum-free medium and stimulated with 100 ng/ml leptin for different times. Cells were washed twice with PBS, fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature, and washed

Table 1. Gene primers for qRT-PCR

Gene	Sequence
14-3-3 ζ	Forward: 5'-GGA TCC ATG GAT AAA AAT GAG CTG GTT-3' Reverse: 5'-ACC AGT ATG TAG GCA GTT TTC-3'
TNF- α	Forward: 5'-CCCAGGCAGTCAGATCATCTTC-3' Reverse: 5'-AGCTGCCCTCAGCTTGA-3'
IL-6	Forward: 5'-GGTACATCCTCGACGGCATCT-3' Reverse: 5'-GTGCCTCTTTGCTGCTTTAC-3'
IFN- β	Forward: 5'-GGATGCAGGAAGGAGATCACTG-3' Reverse: 5'-CGATCCACACGGAGTACTTG-3'
RANTES	Forward: 5'-ATATTCTCCTGGACACCACAC-3' Reverse: 5'-CACTCCAGCTGGGGAAGG-3'
β -actin	Forward: 5'-CTCCTTAATGTCACGCAGGATTTC-3' Reverse: 5'-GTGGGGCGCCCCAGGCACCA-3'

three times with PBS. After that, cells were observed using an Axiophot fluorescence microscope (Zeiss, Germany).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The mRNA of OSCC cell and human tumor tissues was extracted with TRIzol RNA-extraction reagent (Gibco, USA). About 5 μ g of total RNA for each sample was reverse-transcribed into first strand cDNA for qRT-PCR analysis. The qRT-PCR was performed in a final volume of 10 μ l, containing 5 μ l of SsoFast TM EvaGreen Supermix (BIO-RAD, USA), 1 μ l of cDNA (1:50 dilution), and 2 μ l each of the forward and reverse primers (1 mM). The steps in the qRT-PCR were performed as follows: 94°C for 2 min for initial denaturation; 94°C for 20 s, 58°C for 15 s, and 72°C for 15 s; 2 s was used for plate reading for 40 cycles; and a melt curve was generated from 65 to 95°C. The β -actin was used as a quantitative and qualitative control to normalize the gene expression. Data were analyzed using the formula: $R = 2^{-[\Delta Ct \text{ sample} - \Delta Ct \text{ control}]}$. All of the primers used in this experiment are shown in Table 1.

Immunoprecipitation and Western blot analysis

Cells or tumor tissue were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% TritonX-100, 1 mM EDTA, 10 mM β -glycerophosphate, 2 mM

Fig. 1. Analysis of 14-3-3 ζ expression in oral squamous cell carcinoma (OSCC) cells and cancer tissues. 14-3-3 ζ expression was analyzed by qRT-PCR in cancer tissues (A) and cell lines (B); Western blot analysis of 14-3-3 ζ expression in cancer tissues (C) and cell lines (D); β -actin was used as a control. At least three independent experiments were performed. * $p < 0.05$ vs. normal cells, # $p < 0.05$ vs. normal mucosa.

Fig. 2. Analysis of inflammatory cytokines (TNF- α , IL-6, IFN- β , and RANTES) in OSCC cells. (A) 14-3-3 ζ protein expression in OSCC cells after 14-3-3 ζ siRNA transfection; qRT-PCR analysis of TNF- α (B), IL-6 (D), IFN- β (F), and RANTES (H) in OSCC cells. Gene expression was normalized to β -actin mRNA level; Detection of TNF- α (C), IL-6 (E), IFN- β (G), and RANTES (I) in OSCC supernatants by ELISA. At least three independent experiments were performed. * p < 0.05 vs. control.

sodium vanadate and protease inhibitor). Protein concentration was assayed using a micro-BCA protein kit (Pierce, USA). For immunoprecipitation, cell extracts were pre-cleared by Protein A/G Plus beads (Santa Cruz) followed by incubation with primary antibody overnight on a rocker at 4°C. Immune complexes were pulled-down by incubation with Protein A/G Plus beads

for 4 h at 4°C followed by washing twice with lysis buffer containing 0.1% Triton X-100, and twice with lysis buffer without detergent. Bound proteins were eluted by boiling and analyzed by Western blot. For Western blot, 40 μ g of protein per lane was separated by 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane (Amersham Pharmacia, Germany).

Fig. 3. Analysis of IL-6 and RANTES expression in human normal keratinocytes and gingival fibroblasts. (A) 14-3-3 ζ protein expression in human normal keratinocytes and gingival fibroblasts after 14-3-3 ζ vector transfection; Western blot analysis of IL-6 (B) and RANTES (C) expression in human normal keratinocytes and gingival fibroblasts after 14-3-3 ζ vector transfection. Gene expression was normalized to β -actin. At least three independent experiments were performed. * $p < 0.05$ vs. control.

Then, non-specific binding was blocked by incubating with 5% nonfat milk in Tris-buffered-saline with Tween (TBST; 10 mM Tris-HCl, pH of 7.5, 150 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h. Immunodetection of target proteins (14-3-3 ζ , IL-6, RANTES, TNF- α , IL-12, IL-2, IFN- γ , p-Stat3 and total Stat3) and β -actin was performed using mouse monoclonal antibody (1:1500, Santa Cruz), and anti- β -actin (Santa Cruz), respectively. Goat anti-mouse IgG (1:5 000, Sigma, USA) followed by enhanced chemiluminescence (ECL, Amersham Pharmacia, USA) was used.

Statistical analysis

Results are expressed as means $\pm$ SD. Statistical significance was analyzed with one-way factorial ANOVA or Student's two-tailed t -test. A value of $P < 0.05$ was considered statistically significant. All analyses were conducted using SPSS software (SPSS Inc., USA).

RESULTS

14-3-3 ζ is overexpressed in human oral squamous cell carcinoma

To verify the expression of 14-3-3 ζ in oral squamous cell carcinoma, we determined the mRNA level and protein level of 14-3-3 ζ in different cell lines and human tongue cancer tissues. Compared with the normal tissue or normal cells, 14-3-3 ζ was overexpressed in cancer tissue (Figs. 1A and 1C) and carcinoma cells (Figs. 1B and 1D). These results indicate that 14-3-3 ζ plays an important role in tumor development.

14-3-3 ζ inhibits proinflammatory cytokine induction

The 14-3-3 protein has been reported to inhibit Toll-like receptor (TLR)-mediated proinflammatory cytokine induction (Butt et al., 2012). To investigate whether high expression of 14-3-3 ζ in cancer cell might negatively regulate the expression of proinflammatory cytokines, we generated 14-3-3 ζ knockdown Cal27 cells and Tca8113 cells by transfection with 14-3-3 ζ siRNA. Transfection efficiencies were 20-35% for Cal27 cells and 10-15% for Tca8113 cells (Fig. 2A). The mRNA levels of TNF- α , IL-6, IFN- β , and RANTES expression in these cells were analyzed by qRT-PCR. Compared with the control, 14-3-3 ζ knockdown resulted in a marked increase of TNF- α , IL-6, IFN- β , and RANTES levels in these cells (Figs. 2B, 2D, 2F, and 2H). The results were further confirmed via the detection of TNF- α , IL-6, IFN- β , and RANTES proteins in the supernatants by ELISA (Figs. 2C, 2E, 2G, and 2I). To further verify the role of 14-3-3 ζ in down-regulating the expression of cytokines, we next introduced the 14-3-3 ζ adenovirus vector into human normal keratinocytes and gingival fibroblasts prior to stimulation by IFN- γ (50 U/ml) and lipopolysaccharide (LPS; 2.5 μ g/ml). Overexpression of 14-3-3 ζ (Fig. 3A) significantly attenuated IL-6 and RANTES expression in both normal keratinocytes (Fig. 3B) and gingival fibroblasts (Fig. 3C). Overall, these results revealed the inhibitory effect of 14-3-3 ζ on the expression of proinflammatory cytokines.

14-3-3 ζ regulates innate immune and adaptive immune response

Compelling evidence has emerged to show that the immune system is constitutively suppressed by established cancers (Lakshmi Narendra et al., 2013). To investigate the role of 14-3-

Fig. 4. Effect of 14-3-3 ζ on innate and adaptive immune response. (A) 14-3-3 ζ protein expression in Cal27 cells after 14-3-3 ζ siRNA transfection; (B) Western blot analysis of TNF- α and RANTES expression in peritoneal macrophages; (C) Western blot analysis of IL-12 expression in dendritic cells; (D) Evaluation of MHC II expression in dendritic cells by flow cytometry; (E) Detection of proliferation rate in tumor antigen-specific T cell by MTT; (F) Western blot analysis of IL-2 and IFN- γ expression in tumor antigen-specific T cells; Gene expression was normalized to β -actin. At least three independent experiments were performed. * $p < 0.05$ vs. control.

Fig. 5. Examination of the interaction between 14-3-3 ζ and Stat3. (A) Western blot analysis of interactions in Cal27 cells and Tca8113 cells. IgG was used as the control for the bait antibody. (B) Detection of total and phosphorylated protein levels of Stat3 by Western blot analysis. Gene expression was normalized to β -actin. (C) Monitoring Stat3 nuclear translocation by fluorescence microscopy. (D) Cal27 cells were fractionated into cytosolic and nuclear fractions, and equal amounts of protein were analyzed by Western blotting. (E) Evaluation of NF- κ B activity in Cal27 cells and Tca8113 cells. At least three independent experiments were performed. * $p < 0.05$ vs. control.

3 ζ in the regulation of immune response, we disrupted 14-3-3 ζ expression in OSCC with 14-3-3 ζ siRNA sequence (Fig. 4A). Supernatants from these 14-3-3 ζ -knockdown cells elicited obviously increasing levels of TNF- α and RANTES expression in peritoneal macrophages (Fig. 4B). Dendritic cells are responsible for antigen-presenting in adaptive immune response. Conditioned medium of 14-3-3 ζ -knockdown cells also significantly augmented IL-12 (Fig. 4C) and MHC class II (Fig. 4D) expression in dendritic cells. As T cell activation contributes to cell immunity, we next investigated whether interrupting 14-3-3 ζ in OSCC cells might trigger the activation of tumor antigen-specific T cells. Compared with the control, supernatants from 14-3-3 ζ -disrupted OSCC cells promoted proliferation (Fig. 4E) as well as the IL-2 and IFN- γ expression of T cells (Fig. 4F). These results showed that 14-3-3 ζ might negatively regulate innate immune and adaptive immune responses in OSCC.

14-3-3 ζ interacts with Stat3 and promotes its nuclear translocation

As an important transcription factor, Stat3 is constitutively active in many human cancer cells including OSCC, multiple myeloma (MM) and solid tumors (Bromberg et al., 1999, Wang et al., 2012). 14-3-3 ζ might directly contact with Stat3 and modulate its function. At the same time, the role of Stat3 signaling in the immune regulation of cancer cells has also been elucidated (Wang et al., 2004). To verify whether 14-3-3 ζ might interact with Stat3 in OSCC, we detected the expected complex using immunoprecipitation. The expression of Stat3 was detected in the 14-3-3 ζ immune complex and in Cal27 cells and Tca8113 cell lysate but not in the IgG control (Fig. 5A). Stat3 nuclear translocation was also detected in the 14-3-3 ζ -knockdown OSCC (Figs. 5B and 5C). The results showed that the amount of Stat3 distributed in the nucleus was significantly reduced by 14-3-3 ζ siRNA. The results were confirmed by Western blot

Fig. 6. Detection of TNF- α and RANTES expression in Cal27 cells. (A) 14-3-3 ζ and Stat3 protein expression in Cal27 cells after 14-3-3 ζ vector or/and Stat3 siRNA transfection; (B) Western blot analysis of TNF- α and RANTES expression in Cal27 cells transfected with 14-3-3 ζ vector or/and Stat3 siRNA. Gene expression was normalized to β -actin. At least three independent experiments were performed. * $p < 0.05$ vs. Control, # $p < 0.05$ vs. 14-3-3 ζ vector.

analysis of Stat3 expression in the nucleus and cytoplasm after 14-3-3 ζ siRNA transfection. Stat3 protein level was reduced in nucleus but was raised in the cytoplasm (Fig. 5D). Furthermore, a reduction of NF- κ B activity was also observed in 14-3-3 ζ -disrupted OSCC cells (Fig. 5E).

Stat3 is essential for the inhibitory role of 14-3-3 ζ in inflammatory cytokine expression

Given that 14-3-3 ζ -Stat3 complex was constantly formed in OSCC, we hypothesized that Stat3 might mediate the regulatory effect of 14-3-3 ζ on inflammatory cytokine expression. To test this hypothesis, we constructed three OSCC cell models with the transfection of a 14-3-3 ζ vector or/and Stat3 siRNA (Fig. 6A). With the established cell models, the expression of TNF- α and RANTES was analyzed by immunoblotting. Compared with the control, overexpression of 14-3-3 ζ prominently down-regulated the expression of TNF- α and RANTES (Fig. 6B). In contrast, Stat3 knockdown enhanced the protein level of these inflammatory cytokines either with or without the presence of overexpressed 14-3-3 ζ (Fig. 6B). These results indicated that 14-3-3 ζ might regulate inflammatory cytokine expression through Stat3.

DISCUSSION

Expression of 14-3-3 ζ has been reported to occur in many types of malignancies, which include lung cancer (Li et al., 2008), liver cancer (Choi et al., 2011), uterus, breast carcinoma (Neal et al., 2009), stomach cancer (Jang et al., 2004), head and neck squamous cell carcinoma (Lin et al., 2009) and so on. Notably, the expression of 14-3-3 ζ is elevated in breast cancer, lung cancer, multiple myeloma, glioma, meningioma, esophageal cancer, human head and neck squamous cell carcinomas, prostate tumor, oral cancer, hepatoma and pancreatic adenocarcinoma compared with their nonmalignant counterparts (Yang et al., 2012). Our results found that 14-3-3 ζ was overexpressed in OSCC cell lines and cancer tissues, which confirmed the implication of 14-3-3 ζ in carcinogenesis.

Recently, increasing work has been done to elaborate the mechanism behind tumor cell growth and survival induced by 14-3-3 ζ (Neal and Yu, 2010). However, the important role of 14-3-3 ζ in the regulation of interactions between tumor cells and the immune system has not been previously reported. Our studies observed that 14-3-3 ζ knockdown caused elevated proinflammatory cytokine expression in OSCC cell lines. In contrast, introduction of the 14-3-3 ζ vector into normal cells and fibroblasts led to an inhibition of cytokine expression stimulated by IFN- γ and LPS. These results confirmed the effect of 14-3-3 ζ in the modulation of tumor inflammation. Furthermore, conditioned medium from 14-3-3 ζ -knockdown cells up-regulated cytokine synthesis of peritoneal macrophages, boosted the routine activation of dendritic cells and accelerated the normal reaction of tumor-specific T cells. These results revealed the interactions mediated by 14-3-3 ζ between OSCC and the immune system. Our studies also proposed an alternative mechanism, which has been recommended by Ochsenbein et al. (2001), who suggested that established tumors evade immune recognition through either failing to enter lymph nodes or walling themselves off from T cells in lymph nodes.

Activated Stat3 plays a critical role in tumor cell growth, viability, and metastasis in head and neck cancer, as well as in other cancer types (Leeman et al., 2006). Stat3 has been proposed as a therapeutic target for tumor cells as it is a downstream mediator of EGFR and IL6, both of which have been shown to be important in carcinogenesis and angiogenesis (Sriuranpong et al., 2003). In this study, we found that 14-3-3 ζ directly interacted with Stat3 and augmented its nuclear translocation. Stat3 signaling activity is tightly regulated by its interacting proteins and multiple signaling cascades and its prolonged activation is associated with various malignancies, including OSCC (Yu et al., 2007). Many studies have indicated that 14-3-3 proteins are involved in various steps regulating Stat3 activity, including PKC (Saurin et al., 2008), Raf1 (Dhillon et al., 2009), MEK1/2 (Fanger et al., 1998), and PP2A (Sun et al., 2007). Moreover, 14-3-3 ζ may serve as a molecular stabilizer, which contributes to serine phosphorylation and converts Stat3 from the form with lower activity to that with higher activity (Zhang et al., 2012).

Wang et al. (2004) demonstrated that Stat-3 can mediate immune evasion by blocking both the production and sensing of inflammatory signals by multiple components of the immune system. Our studies have shown that cytokine expression inhibition caused by 14-3-3 ζ was abated by the knockdown of Stat3, which further confirms the hypothesis that Stat3 might play an essential role in the regulation of tumor inflammation.

Collectively, we demonstrated that 14-3-3 ζ regulates tumor inflammation and immune response through Stat3 signaling in

OSCC. 14-3-3 ζ is overexpressed in OSCC cell lines and cancer tissues. In addition, 14-3-3 ζ directly interacts with Stat3, which has an essential role in modulating tumor inflammation, and targeting 14-3-3 ζ may be challenging as 14-3-3 ζ regulates many important proteins that are essential for homeostasis. Thus, more effort should be made to find a promising way for the prevention and treatment of OSCC.

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