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Original Article

The enhancement of TRAIL-induced apoptosis sensitivity of oral cancer Ca9-22 cells by over-expressing RASSF5A

Wei Liu[1], Youyang Zheng[1], Ben Chen[1], Qiongdan Zheng[1], Zhuojin Shi[2]

[1]*Department of Stomatology, 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China;* [2]*School of Stomatology, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China*

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Abstract: Ras association domain family protein 5A (RASSF5A) is one member of Ras receptor family. It can phosphorylate FOXO3a via activating MST1 kinase to up-regulate BIM expression and facilitate apoptosis. Down-regulation of RASSF5A is correlated with tumor necrosis factor related apoptosis inducing ligand (TRAIL) induced apoptosis resistance. This study thus investigated if RASSF5A regulates TRAIL-induced apoptosis sensitivity in oral cancer (OC) cells. 39 OC patients were collected from tumor and adjacent tissues. Expression of RASSF5A, p-FOXO3a and BIM was measured. *In vitro* cultured KB and Ca-22 cells were treated with TRAIL for 48 hours, followed by MTT and flow cytometry for quantifying proliferation and apoptosis. Expressions of RASSF5A, p-FOXO3a and BIM were also compared. Ca9-22 cells were treated with RASSF5A-pMD18 or controlled plasmids for measuring RASSF5A, p-FOXO3a and BIM expression, along with proliferation and apoptosis. OC tissues had lower RASSF5A, p-FOXO3a and BIM expression than adjacent tissues. Gene expression levels, cell proliferation inhibition and apoptosis rate were all lower in Ca9-22 cells compared to KB. Over-expression of RASSF5A significantly enhanced gene expression, phosphorylation of FOXO3a and inhibited proliferation, whilst BIM expression and cell apoptosis were potentiated. RASSF5A down-regulation is related with TRAIL resistance of Ca9-22 cells. Over-expression of RASSF5A significantly enhances gene expression, FOXO3a phosphorylation, expression of pro-apoptotic protein BIM and cell apoptosis.

Keywords: RASSF5A, TRAIL, oral cancer, cell apoptosis, cell proliferation

Introduction

Oral cancer (OC) is one common malignant tumor in head-neck skin, with relatively higher incidence and mortality worldwide. Squamous cell carcinoma is the major pathological type of OC [1]. Tumor necrosis factor related apoptosis inducing ligand (TRAIL) selectively induce tumor cell apoptosis but has no significant effects on normal cells, endowing it wide application in clinical treatment of tumors [2]. TRAIL binds onto membrane death receptor 4 (DR4) or DR5 to form ligand-receptor trimer for recruiting downstream molecules to form death-inducing signaling complex (DISC) to initiate Caspase cascade reaction [3]. Although TRAIL has apoptotic induction effects on most tumor cells, certain cancer cells, however, displays insensitivity for TRAIL induced apoptosis. Such resistance and related drug insensitivity largely limit clinical application of TRAIL [4]. Ras association domain family protein 5A (RASSF5A), also named as NORE1A, is one important member of Ras association domain family [5]. RASSF5A can bind onto mammalian sterile 20-like (MST1) kinase to facilitate its nuclear trafficking, where it can initiate transcription and translation of downstream target gene Bcl-2 interacting mediator of cell death (BIM) via phosphorylating pro-apoptotic factor FOXO3a, thus accelerating cell apoptosis [6]. Down-regulation of RASSF5 has been shown to be related with occurrence of multiple tumors including esophageal carcinoma [7], neuronal tumor [8] and pulmonary carcinoma [9]. Lower RASSF5 expression has also been shown to be related with DR ligand TNF-α induced apoptosis impeding [10]. Volodko et al showed the relationship between lower RASSF expression and lesion in head-neck [11]. Imari et al revealed significantly low-

ered RASSF2 expression in squamous carcinoma tissues of OC [12]. This study thus investigated if RASSF5A plays a role in affecting sensitivity of OC cells against TRAIL-induced apoptosis and OC pathogenesis.

Materials and methods

Reagent and materials

TRAIL resistant cell line HCCLM3 and sensitive cell line KB were purchased from Japanese Collection of Research Bio-resources (Japan). DMEM was purchased from Gibco (US). Fetal bovine serum (FBS) was purchased from Gemini (US). Penicillin-streptomycin was purchased from Gibco (US). RNA extraction reagent was purchased from OMEGA (US). ReverTra Ace aPCR RT Kit and SYBR Green dye were purchased from Toyobo (Japan). Rabbit anti-RASSF5A and anti-p-FOXO3a were purchased from Abcam (US). Mouse anti-BIM was purchased from Abnova (US). Anti-cleaved caspase-3 was purchased from CST (US). HRP conjugated anti-mouse/rabbit secondary antibody was purchased from Jackson ImmunoResearch (US). Caspase-9 and Caspase-3 activity assay kits were purchased from Beyotime (China). Annexin V/PI apoptosis kit was purchased from Sangon (China). Phosphate calcium based transfection reagent was purchased from Beyotime (China).

Clinical information

A total of 39 OC patients who received treatment in Department of Stomatology, 2nd Affiliated Hospital, School of Medicine, Zhejiang University from September 2015 to June 2016 were recruited. Tumor samples and adjacent tissues were collected. There were 21 males and 18 females, aging between 41 and 76 years (average age = 61.8 years). All patients have not received surgical resection before, or other medical treatment.

The study was approved by 2nd Affiliated Hospital, School of Medicine, Zhejiang University Ethics Committee and all the subjects had signed informed consent.

Cell culture and TRAIL treatment

Human OC cell line Ca9-22 and KB were incubated in DMEM medium containing 10% FBS and 1% streptomycin-penicillin. Cells were then treated with TRAIL at 0, 20, 40 and 80 ng/mL for 48 h. Cells at satisfactory growth status were collected for detecting indexes.

MTT for cell proliferation activity

Cells were seeded into 96-well plate. After fully attached for 24 h, cells were then treated with TRAIL at 0, 20, 40 and 80 ng/mL for 48 h. 4 h before quenching incubation, 10 μl MTT solution (5 mg/ml) was added into each test well. After 4 h continuous culture, the original culture medium was discarded, with twice PBS washing. 150 μl DMSO was the added into each well vortex until the complete resolving of crystal. Absorbance values at 450 nm (A450) were measured by a microplate reader. Each treatment group included five replicates. Relative proliferation activity = (A450 in treatment group - A450 in blank control group)/A450 in untreated group X 100%.

Construction of over-expression plasmids

RASSF5A gene was amplified using cDNA as the template and specific primers (Forward, 5'-GACGA ATTCA TGGCA AAGCA ACCT-3'; Reverse, 5'-GACGT CGACT TAATC AGGTG GAAG-3'). After gel extraction and purification, PCR products were ligated into pMD18-T vector to transform BJ5183 competent cells. By positive selection using ampicillin-containing culture medium, positive clone was further amplified to extract recombinant plasmid. DNA sequencing was then performed to confirm the correct insertion of targeted fragments.

Cell transfection and grouping

Phosphate calcium based method was used to transfect empty plasmid (Blank-pMD18-T), negative controlled plasmid (Scramble-pMD-18-T) and over-expression plasmid (RASSF5A-pMD18-T) into Ca9-22 cells. G418 culture medium was used to screen positive clones with stable transfection. Ca9-22 cells were treated with 100 ng/ml TRAIL and were divided in three groups: Blank-pMD18-T group, Scramble-pMD18-T group, and RASSF5A-pMD18 group. 72 h later cells were collected for assay.

qRT-PCR for gene expression

OMEGA reagent was used to extract total RNA, which was kept at -80°C for further use. ReverTra Ace qPCR RT Kit synthesized cDNA

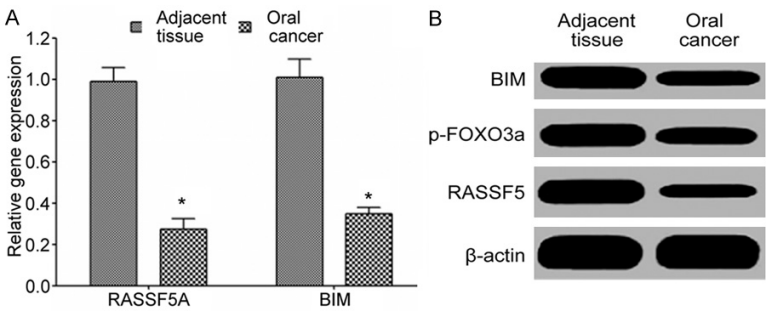

Figure 1. Lower RASSF5A, p-FOXO3a and BIM expressions. A. qRT-PCR for RASSF5A and BIM gene expression; B. Western blot for protein expression. *P<0.05 compared to adjacent tissues.

from RNA by reverse transcription. Using cDNA as the template, PCR amplification was carried under TaqDNA polymerase. Primer sequences used were: $RASSF5AP_F$: 5'-GGGCA TGAAA CTGAG TGAAG A-3'; $RASSF5AP_R$: 5'-TGGCA TCATA GATGG ACTGG G-3'; $BIMP_F$: 5'-ATCTC AGAGC AATGG CTTCC-3'; $BIMP_R$: 5'-ATTCG TGGGT GGTCTT CG-3'; β-$actinP_F$: 5'-GAACC CTAAG GCCAA C-3'; β-$actinP_R$: 5'-TGTCA CGCAC GATTT CC-3'. In a 10 μL system, we added 4.5 μL SYBR Green Mixture, 1.0 μL forward primer, 1.0 μL reverse primer, 1.0 μL cDNA, and 2.5 μL ddH_2O. The reaction conditions were: 95°C for 5 min, followed by 95°C 15 sec and 60°C 60 s. 40 cycles were performed on Bio-Rad CFX96 fluorescent quantitative PCR cycler for collecting data.

Western blot

Total proteins were extracted by routine methods. 50 μg protein samples were separated in 10% SDS-PAGE (3 h), and were transferred to PVDF membrane (wet method, 1.5 h). The membrane was blocked in 5% defatted milk powder for 60 min, followed by primary antibody (anti-RASSF5A at 1:300, anti-p-FoxO3a at 1:100, anti-BIM at 1:200, anti-Cleaved Caspase-3 at 1:200, or anti-β-actin at 1:800) incubation at 4°C overnight. By PBST washing (3 times), HRP-labelled secondary antibody (anti-mouse or anti-rabbit at 1:5000 dilution) was added for 60 min incubation under room temperature. After PBST rinsing for three times, ECL reagent was added for 3 min dark incubation. The membrane was then exposure in dark and scanned for analysis of protein band density using Quantity One software.

Spectrometry for caspase-3 activity

Standard dilutions of 0, 10, 20, 50, 100 and 200 μM pNA were prepared. Absorbance values at 405 nm wavelength (A405) were measured by a microplate reader to plot a standard curve with pNA concentration against A405 value. Culture medium was collected. Attached cells were digested and were collected. 100 μL lysis buffer was added for every 2×10^6 cells. Cells were lysed at 4°C for 15 mn, and were centrifuged at 18000 g with 4°C for 10 min. The supernatant was saved for further use. Ac-DEVD-pNA was placed on ice, mixed with buffer and test samples, with 10 μL Ac-DEVD-pNA. The mixture was incubated at 37°C for 120 min. A405 value was measured when color changed significantly.

Flow cytometry for cell apoptosis

Cells were collected in trypsin, and were washed twice in PBS. 100 μL Binding Buffer was used to re-suspend cells. The mixture was then mixed with 5 μL Annexin V-FITC and 5 μL PI, and was incubated in dark for 15 min. FC 500MCL flow cytometry (Beckman Coulter) was used to test cell apoptosis.

Statistical analysis

SPSS20.0 was used to collect and analyze all data, in which measurement data were presented as mean ± standard deviation (SD). The comparison of measurement data between two groups was performed by two-sample independent t-test. A statistical significance was defined when $P<0.05$.

Results

Significant decrease of RASSF5A, p-FOXO3a and BIM expressions in OC tissues

qRT-PCR results showed significantly lower expression level of RASSF5A and BIM mRNA in OC tumor tissues (**Figure 1A**). Western blot results showed that, compared to adjacent tissues, OC tissues had significantly lower RASSF5A protein expression, plus weakened phosphorylation level of downstream FOXO3a, and lower expression of pro-apoptotic protein BIM (**Figure 1B**). These results suggested the

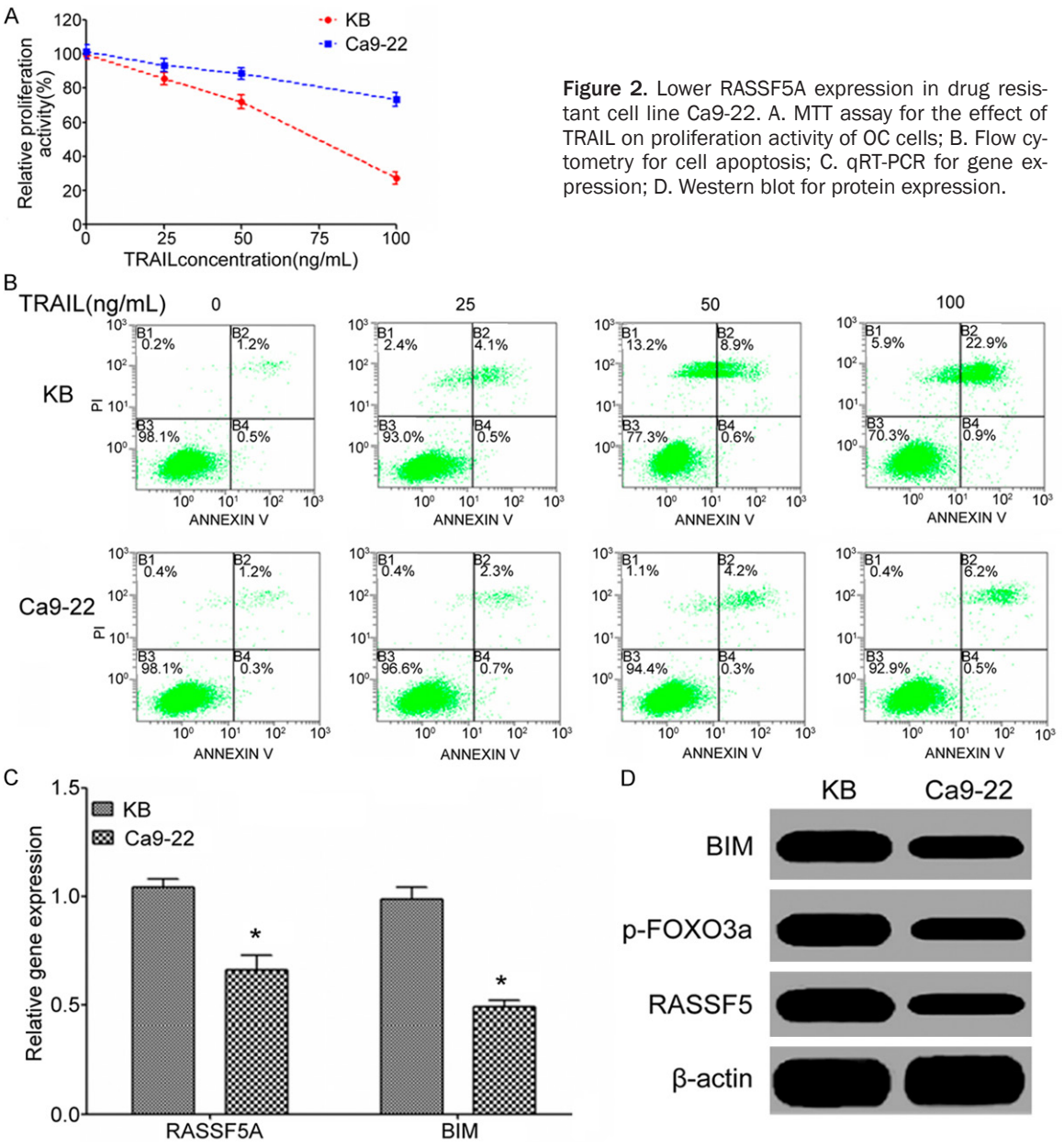

Figure 2. Lower RASSF5A expression in drug resistant cell line Ca9-22. A. MTT assay for the effect of TRAIL on proliferation activity of OC cells; B. Flow cytometry for cell apoptosis; C. qRT-PCR for gene expression; D. Western blot for protein expression.

potential relationship between lower RASSF5A expression and pathogenesis of OC.

Lower RASSF5A expression in drug resistance cell line Ca9-22

Different concentrations of TRAIL significantly inhibited proliferation activity of KB cells. Under high concentration (100 ng/mL), the proliferative activity of KB cell was decreased to 25% of original level. Ca9-22 cell had weaker inhibition of proliferation compared to that in KB cells, as 100 ng/mL TRAIL only decreased proliferative activity of Ca9-22 cells to about 80%, indicating certain drug resistance of Ca9-22 cells against TRAIL (**Figure 2A**). Flow cytometry results showed that different concentrations of TRAIL could significantly induce KB cell apoptosis, whilst Ca9-22 cells showed insensitivity for TRAIL-induced apoptosis (**Figure 2B**). qRT-PCR results showed significantly lower mRNA expression of RASSF5A and BIM in Ca9-22 cells compared to KB cells (**Figure 2C**). Western blot further showed lower RASSF5A protein expression in in Ca9-22 cells compared to KB cells, plus weakened phosphorylation of down-

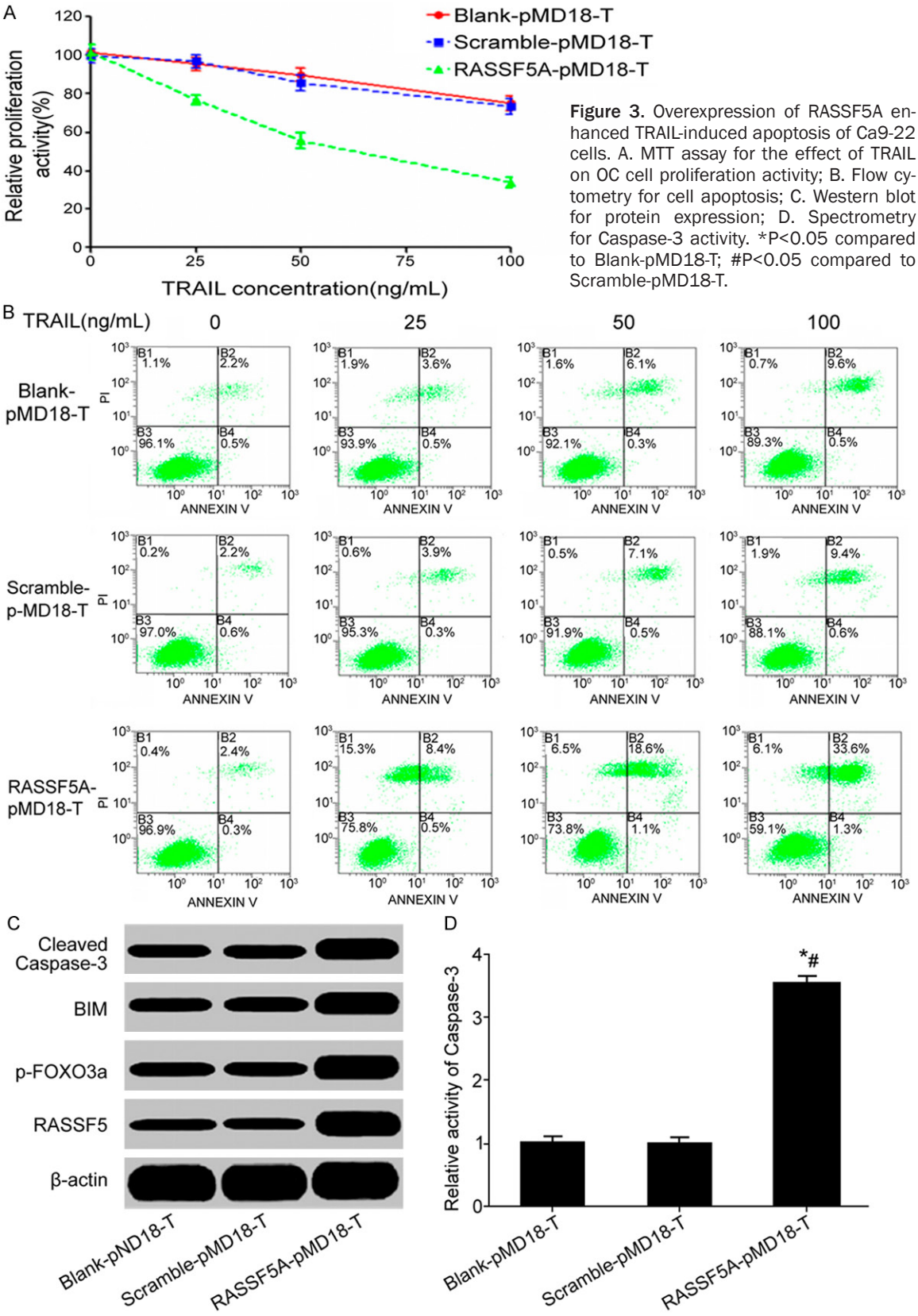

Figure 3. Overexpression of RASSF5A enhanced TRAIL-induced apoptosis of Ca9-22 cells. A. MTT assay for the effect of TRAIL on OC cell proliferation activity; B. Flow cytometry for cell apoptosis; C. Western blot for protein expression; D. Spectrometry for Caspase-3 activity. *P<0.05 compared to Blank-pMD18-T; #P<0.05 compared to Scramble-pMD18-T.

stream FOXO3a and lower pro-apoptotic protein BIM (**Figure 2D**). These results indicated the potential role of RASSF5A down-regulation in TRAIL resistance of Ca9-22 cells.

Over-expression of RASSF5A enhanced TRAIL-induced Ca9-22 cell apoptosis

MTT assay showed that, compared to those transfected with empty plasmid, Ca9-22 cells transfected with scrambled plasmids had no significant effects on TRAIL-induced proliferation activity, whilst over-expression of RASSF5A plasmid transfection significantly enhanced the inhibition of TRAIL on proliferation activity of Ca9-22 cells (**Figure 3A**). Flow cytometry showed that over-expression of RASSF5A significantly enhanced induction of Ca9-22 cell apoptosis by TRAIL (**Figure 3B**). Further assay showed that over-expression of RASSF5A enhanced its expression in Ca9-22 cells, potentiating phosphorylation level of downstream FOXO3a proteins, and increasing expression of pro-apoptotic protein BIM, cleaved Caspase-3 expression and its activity (**Figure 3C** and **3D**).

Discussion

It is estimated that OC occupies about 2%~4% of all malignant tumors, and 10%~20% cancers in head-neck region. Recently, OC patients number is rapidly increasing, as more than 280000 people are newly diagnosed as OC worldwide [13]. Both incidence and mortality of OC are among the top ten of malignant tumors. Worldwide, incidence of OC is 12.0 per 100000, whilst mortality is 5.1 per 100000 [13]. American Society of Cancer reported 45780 patients newly diagnosed as OC in US, including 32670 males and 13110 females [14]. 8650 people died from OC in the year 2015, including 6010 males and 2640 females. In China, OC incidence is as high as 48.1 per 100000, plus 22.1 per 100000 mortality [15]. In addition, post-op recurrent rate is also higher reaching 36.6%~38.3% [16]. With advancement in medical science, combined treatment is now feasible but without significant improvement for patient prognosis, as 5-year survival rate is only 50% post-up, with 60~65 months median survival time [17].

TRAIL is one newly member of tumor necrosis factor (TNF) superfamily recently been discovered. It can bind with death domain (DD) at C terminus of Fas-associated death domain (FADD) via DD of cytoplasmic segment of DR4 or DR5 [4]. FADD can bind with Proeaspase-8 via its N terminal death effector domain (DED) to form DISC including DR4/DR5-FADD-Procaspase-8, to facilitate auto-cleavage of Procaspase-8 to generate active Caspase-8 for sequential activating of downstream executing proteins Caspase-3, -6 and -7 to induce cell apoptosis [18]. Ras association domain family is one group of receptor proteins containing Ras association structural domains, including multiple protein subunits from RASSF1 to RASSF10. RASSF5A, also names as NORE1A, is the product of longest splicing mRNA of RASSF5 gene. This protein consists of one RAS association domain and one C-terminal SARAH structural domain. It can participate in inducing Ras signal pathway cascade reaction via binding with GTP forms or other RAS-like GTPase such as Rap1, M-Ras, R-Ras/R-Ras3, thus being involved in the regulation of multiple biological processes including cell proliferation, differentiation, tissue/organ development and oxidative stress response [10]. RASSF5A can bind with mammalian sterile 20-like MST1 kinase via its C terminal structural domain to facilitate nuclear transportation of MST1 from cytoplasm. The nuclear localized MST1 can phosphorylate apoptotic transcription factor FOXO3a, up-regulate transcription and translation of downstream target gene BIM, thus initiating cell apoptosis pathway [4, 19, 20]. As one pro-apoptotic factor, BIM can interact with another apoptotic protein Bax via its BH3-only structural domain, to form oligomer of the latter and insertion into mitochondrial membrane, thus elevating membrane permeability, loss of transmembrane potential, releasing cytochrome C (Cytc), thus activating mitochondria-dependent cell apoptosis transduction pathway [21, 22]. Volodko et al showed the correlation between down-regulation of RASSF and swelling of head-neck region [11]. Imai et al showed significantly lower expression of RASSF2 from RASSF protein family in OC squamous carcinoma tissues [12]. Down-regulation of RASSF5 has also been demonstrated to be related with impeding of DD ligand TNF-α induced apoptosis [10]. This study thus investigated if RASSF5A played a role in affecting TRAIL-induced apoptotic sensitivity of OC cells.

Test results showed significantly lowered RASSF5A expression in OC tumor tissues, which also had weakened phosphorylation level of downstream FOXO3a, and depressed expression of pro-apoptotic protein BIM. Compared to TRAIL-sensitive KB cells, Ca9-22 cells had

lower expression of RASSF5A, weakened phosphorylation of downstream FOXO3a, and lower pro-apoptotic protein BIM. These studies showed that down-regulation of RASSF5A potentially played a role in TRAIL resistance of OC cells and pathogenesis. Further assay showed that over-expression of RASSF5A significantly enhanced its expression in Ca9-22 cells, and facilitated phosphorylation of downstream FOXO3a protein, plus higher BIM expression, potentiated Caspase-3 activity and apoptosis, plus inhibition of cell proliferation potency. During the initiation of apoptotic signal, RASSF5A mainly induces mitochondria dependent apoptotic pathway via activating downstream FOXO3a and BIM. TRAIL mainly induces Caspase-8 dependent cell apoptosis pathway via death receptor. Previous study showed the close relationship between mitochondria induced apoptosis and death receptor induced apoptosis [23], as Caspase-8 in death receptor pathway can directly activate Caspase-3 for programmed cell death, and can cleave Bcl-2 family member via decreasing potential of mitochondrial inner member to release cytochrome C, which further bind with apoptotic protease activating factor-1 (Apaf-1) to recruit and activate Caspase-9, which initiate mitochondria dependent cell apoptotic pathway. Therefore, death receptor induced apoptosis signal pathway has synergistic and amplified effects in conjunction with mitochondrial dependent apoptotic pathway. This study observed that over-expression of RASSF5A significantly facilitated TRAIL-induced apoptosis of OC cell line Ca9-22, probably related with enhanced mitochondrial dependent apoptotic pathway in drug resistant Ca9-22 cells after RASSF5A over-expression, and consequently synergistic and amplified effects with death receptor induced apoptotic pathway, although leaving detailed functional mechanism to be further illustrated. Park et al showed that siRNA interference on RASSF5 expression significantly decreased the apoptosis of mouse embryonic fibroblasts (MEFs) induced by death receptor ligand TNF-α or TRAIL, thus suppressing the sensitivity of TRAIL-induced apoptosis [10]. Similar effects can be achieved via siRNA interference on RASSF5 downstream protein MST1. Moreover, MEFs with knockout of RASSF5 gene showed malignant transformation at early stage of *in vitro* passage culture. This study showed that over-expression of RASSF5A could facilitate TRAIL-induced OC cell apoptosis, probably sharing similar mechanisms as reported by Park et al [10].

Conclusion

Down-regulation of RASSF5A is correlated with TRAIL resistance of Ca9-22 cells. Over-expression of RASSF5A significantly enhances RASSF5A expression in Ca9-22 cells, and potentiates phosphorylation of downstream FOXO3a, increases expression of pro-apoptotic protein BIM and cell apoptosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhuojin Shi, School of Stomatology, Zhejiang Chinese Medical University, No. 548, Binwen Road, Hangzhou, Zhejiang, China. Tel: +86-0571-86633321; Fax: +86-0571-86633080; E-mail: tingtingwanghj@163.com

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