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Original Article

Expression of MICA in oral squamous carcinoma cells and its effect on NK cells

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Abstract: Objective: This study aims to observe the expression of MHC-class I chain related protein A (MICA) in oral squamous carcinoma cell and explore its effects on NK cells. Methods: Normal oral mucosa epithelial cell line NOK and oral squamous carcinoma cell lines OEC-M1, SAS and SCC-25 were used in this study. MICA expression in the cells was detected by western blotting and RT-PCR methods, sMICA was detected by ELISA method. The cells were transfected by pEGFP-MICA and pEGFP-NC respectively using Lipofectamine 2000 kit. The transfected cells were co-cultured with NK92 cells. Killing activity of NK92 cells was detected by LDH release method and NKG2D was detected by Flow cytometry. ADAM10 and ADAM17 were detected by ELISA method. Results: MICA expression in OEC-M1, SAS and SCC-25 cells were lower than that of NOK cells (P<0.01), sMICA levels in OEC-M1, SAS and SCC-25 cells were higher than that of NOK cells (P<0.01). Over-expression of MICA in SCC-25 cells could significantly increase the killing activity of NK92 cells (P<0.01), up-regulate NKG2D (P<0.01) and decrease ADAM10 and ADAM17 contents (P<0.01). Conclusions: MICA expressed lowly in oral squamous cell carcinoma cells, over-expression of MICA could significantly increase the killing activity of NK92 cells, which could be related with the regulation of ADAM.

Keywords: MHC-class I chain related protein A (MICA), oral squamous carcinoma cell lines, NK cells

Introduction

Oral squamous cell carcinoma (OSCC) is mainly treated by operation supplemented by chemotherapy method, however, the facial morphology of patients could be severely affected which leading to disorder of chewing, swallowing, breathing, language, etc [1, 2]. OSCC incidence increased these years and 1/3 patients recrudesced and died of metastasis [3]. It was confirmed that the immune function of OSCC patients was low which was closely related to the development of OSCC [4, 5].

Major histocompatibility complex class I chain-related protein A (MICA) was transmembrane protein encoded by MHC gene. It was the major ligand of natural killer cell group 2 member D (NKG2D) and bound with NKG2D specially to activate the immune effector cells. It was found that MICA abnormally expressed in many epithelial and non epithelial tumor cells and was considered to be a kind of tumor associated antigens [6, 7]. In this study we observed the expression of MICA in OSCC cells and NOK cells and explored its effects on NK cells. This was helpful to further elucidate the molecular mechanism of the development and prognosis of OSCC, which had important significance in the improvement of survival rate of patients with OSCC.

Materials and methods

Cell culture

Normal oral mucosa epithelial cell line NOK, oral squamous carcinoma cell lines OEC-M1, SAS and SCC-25 and NK92 cells were supplied by Shanghai Jiaotong University School of Medicine. Cells were cultured in RPMI1640 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C with 5% CO_2.

Cell transfection

Cells were seeded in 6-well plates and cultured at 37°C with 5% CO_2 with 6 × 10^4 cells/ml. pEGFP-NC and pEGFP-MICA were transfected using

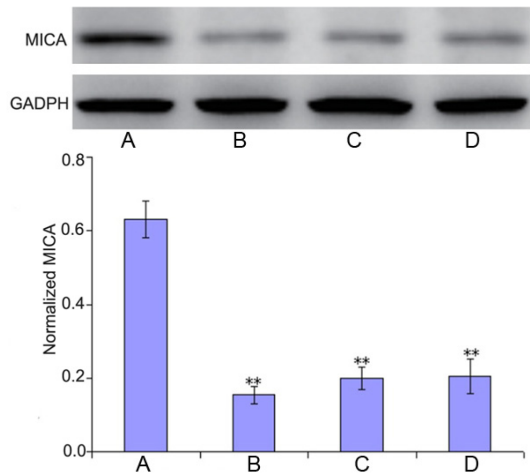

Figure 1. Expression levels of MICA in NOK, OEC-M1, SAS and SCC-25 cells. A: NOK; B: OEC-M1; C: SAS; D: SCC-25. Compared with NOK, **$P<0.01$.

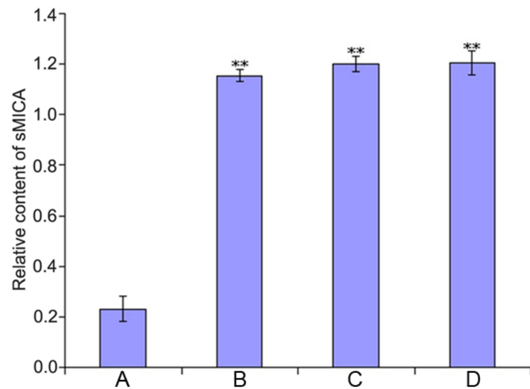

Figure 2. Content of sMICA in NOK, OEC-M1, SAS and SCC-25 cells. A: NOK; B: OEC-M1; C: SAS; D: SCC-25. Compared with NOK, **$P<0.01$.

lipofectamine 2000 according to the manual and cultured for 72 h.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol reagent Kit (Invitrogen) according to the manufacturer's protocol. The RNA was further purified using an RNeasy cleanup kit (Qiagen, Valencia, CA). 1 μg total RNA was subjected to reverse transcription using reverse transcription system (Promega). Real-time PCR were performed using SYNBR Green PCR Master Mix (Qigen). Primers were as follows: MICA Forward: 5'-AGCCCCAC-AGTCTTCGTTATA-3', Reverse: 5'-CAAGGTCTGA-GCTCTGGAGGA-3'; Glyceraldehyde 3-phosphate dehydrogenase (GADPH) Forward: 5'-AATC-CCATCACCATCTTCCA-3', Reverse: 5'-CCTGCTT-CAACCACCTTCTTG-3'. GADPH gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantifications of target genes mRNA was performed using the $2^{-\Delta\Delta Ct}$ method. The PCR products underwent electrophoresis on 1.0% agarose gel.

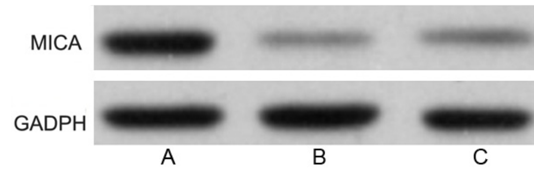

Figure 3. Western blotting results of MICA. A: Control group; B: pEGFP-NC group; C: pEGFP-MICA group.

Western blotting analysis

Proteins were extracted from cultured cells with RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.2% Na-deoxylate 1x protease cocktail) (Sigma-Aldrich, St. Louis, MO). The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manual. Samples were separated on 12% SDS gels, transferred to PVDF membranes (GE Healthcare Life Sciences, Buckinghamshire, UK). After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate primary antibodies. Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to GAPDH.

Detection of NK cell killing activity

NK cell killing activity was detected with LDH releasing method. The cells were cultured in RPMI1640 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C with 5% CO_2 for 48 h and they were divided into four groups: Test well (A): OSCC cells and NK92 cells

Table 1. The effects of MICA over-expression in OSCC cells on the killing activity of NK92 cells ($\overline{X}\pm S$, %)

Group	NK92 killing activity	NKG2D content
Control	28.54±5.04	30.27±4.75
pEGFP-NC	29.38±3.24	31.97±3.52
pEGFP-MICA	49.08±5.28**	51.48±2.89**

Compared with control group, **P<0.01.

(1:10); Natural release well (B): OSCC cells and RPMI1640 medium (1:1); Maximum release well (C): OSCC cells and 20% SDS; Control well (D): NK92 cells and RPMI1640 medium (1:1). The supernatant was put into 96-well plate and LDH substrate was added into them according to the manual. OD values were determined with microplate reader. Killing activity (%) = (OD of A-OD of B-OD of D)/(OD of C-OD of D) × 100%.

Flow cytometry analysis

NKG2D expression in NK92 cells was detected by Flow cytometry. The cells in above test well were collected and added in NKG2D antibody and IgG1 respectively, they reacted at room temperature for 30 min. They were detected by Flow cytometry and analyzed with CellQuestPro software.

ELISA assay

sMICA in NOK, OEC-M1, SAS and SCC-25 cells before transfection were determined by ELISA kit according to the manual. ADAM10 and ADAM17 contents in NOK, OEC-M1, SAS and SCC-25 cells after transfection were determined by ELISA kit according to the manual.

Statistical analysis

The data are expressed as mean ± SD and analyzed with SPSS 17.0 software, t test was used to evaluate the differences among groups. A value of $P<0.05$ was taken to denote statistical significance.

Results

MICA expression

The expression levels of MICA in NOK, OEC-M1, SAS and SCC-25 cells were shown in **Figure 1**. It showed that MICA in OSCC cells were lower than that of NOK cells (0.15±0.02, 0.19±0.03, 0.20±0.04 vs. 0.63±0.05) (P<0.01).

Determination of sMICA

Determination of sMICA content in different cells was shown in **Figure 2**. It showed that sMICA content in OEC-M1, SAS and SCC-25 cells were significantly higher than that of NOK cells (1.15±0.03, 1.19±0.03, 1.20±0.05 vs. 0.23±0.05) (P<0.01).

Western blotting results

MICA in OSCC cells after transfection was detected using western blotting method and the results were shown in **Figure 3**. It showed that MICA increased after transfection, which suggested that over-expression vector was constructed successfully.

The effects of MICA over-expression in OSCC cells on the killing activity of NK92 cells

The effects of MICA over-expression in OSCC cells on the killing activity of NK92 cells were shown in **Table 1** and **Figure 4**. We found that over-expression of MICA in OSCC cells significantly increased NKG2D content in NK92 cells and the killing activity of NK92 cells (P<0.01).

The effects of MICA over-expression in OSCC cells on ADAM10 and ADAM17

As shown in **Table 2**, ADAM10 and ADAM17 content significantly decreased in OSCC cells with MICA over-expression (P<0.01).

Discussion

MICA is a highly glycosylated transmembrane glycoprotein, it presents as membrane binding type MICA and soluble MICA (sMICA) forms in the body. The first form activated the immune function of NK cells by binding with NKG2D. sMICA induced internalization and degradation of NKG2D, which leading to tumor escape immune response. It was confirmed that MICA abnormally expressed in OSCC cells and was closely related to the occurrence and development of OSCC [8]. In this study we also found that MICA expression was lower in OSCC cells than that of NOK cells. Tamaki found that sMICA levels in OSCC patients were higher than that in normal people [9]. In this study we found that sMICA in OSCC cells was higher than that of NOK cells, it was consistent with the above results.

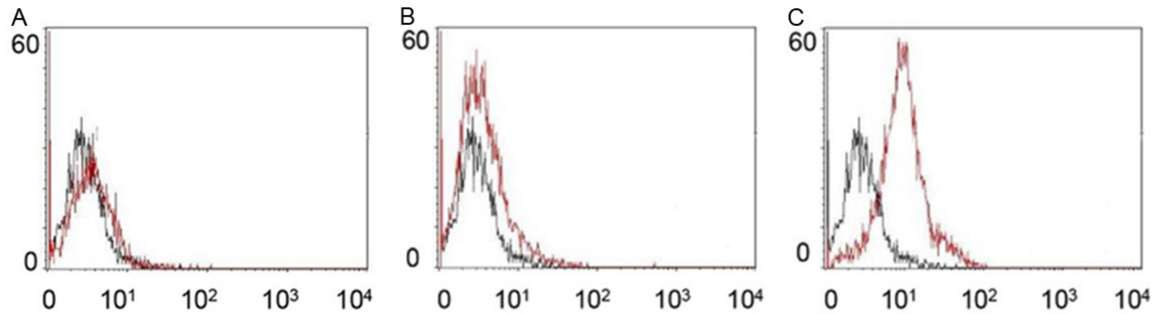

Figure 4. Results of flow cytometry analysis. A: Control group; B: pEGFP-NC group; C: pEGFP-MICA group.

Table 2. The effects of MICA over-expression in OSCC cells on ADAM10 and ADAM17 ($\bar{X}\pm S$)

Group	ADAM10 (pg/ml)	ADAM17 (pg/ml)
Control	183.45±14.35	208.37±18.62
pEGFP-NC	179.39±10.49	207.93±20.48
pEGFP-MICA	58.48±5.5**	65.35±6.48**

Compared with control group, **P<0.01.

Busche found that MICA could enhance NK cell killing activity [10]. Over-expression of MICA in NK cells and CD8+T cells could significantly improve its toxicity to corneal epithelial cell [11]. Overexpression of MICA could significantly improve the sensitivity of tumor cells to NK and other immune cells [12]. In this study we found that over-expression of MICA in OSCC cells could promote the killing activity of NK cells.

It was confirmed that MICA-NKG2D played an important role in the regulation of NK cells in tumor cells, sMICA mediated tumor immune escape by downregulation of NKG2D [13]. The decrease of sMICA content had important effect on the improvement of immune surveillance function of NKG2D cells [14]. NKG2D was a NK cell activating receptor and widely expressed in surface of NK cells, CTL cells, γδT cells, CD8+T cells and activated macrophages. Expression of NKG2D in PBMCs of OSCC patients was lower than that of normal people and was closely related with tumor size [15]. It could significantly improve the anti tumor immune response by improving the expression of MICA and its receptor NKG2D [16]. Up-regulation of MICA and NKG2D could significantly improve the target toxicity of NK cells to tumor cells [17]. In this study we found that over-expression of MICA in OSCC cells could decrease sMICA content and up-regulate NKG2D expression in NK cells. It suggested that increase of MICA in OSCC cells could enhance the killing activity of NK cells to OSCC cells.

It was found that ADAMs could degrade MICA in tumor cell membrane surface to make it fall off and reduce antigenicity, which causing NKG2D in NK cells failing to recognize tumor cells and reduce the attack and killing effect to tumor cells [18, 19]. ADAMs is a transmembrane protease with the function of proteolytic enzymes, it could hydrolyze growth factor, cell factor and other membrane surface protein and release them to their extracellular function area. Waldhauer found that ADAM10 was involved in the abscission of MICA [19]. Chitadze [20] found that ADAM10 and ADAM11 could promote the production of sMICA/B in pancreatic cancer, prostate cancer and other cancer cells, the inhibition of ADAM could decrease the release of MICA/B. In this study we found that over-expression of MICA in OSCC cells could decrease ADAM10 and ADAM17 content.

In a word, MICA expressed lowly in OSCC cells, over-expression of MICA could significantly increase the killing activity of NK92 cells, which could be related with the regulation of ADAM.

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Disclosure of conflict of interest

None.

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