

## RESEARCH COMMUNICATION

# Clinico-Pathological Significance of MHC-I Type Chain-associated Protein A Expression in Oral Squamous Cell Carcinoma

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### Abstract

The current research concerns the clinicopathological significance of MHC class I chain-related protein A (MICA) expression in oral squamous cell carcinomas (OSCCs). The expression and location of MICA protein in 14 normal oral mucous and 45 cancerous and para-cancerous tissues were assessed by immunohistochemistry and levels of MICA mRNA expression in 29 cancerous and para-cancerous tissues were determined by the real-time polymerase chain reaction. Data were analyzed with the SPSS16.0 software package. MICA was found to be located in the cytoplasm and plasma membrane. Expression was higher in para-cancerous than in cancerous tissues ( $P < 0.05$ ). However, no statistical difference was found between the following: 1) para-cancerous tissue with normal mucosa; 2) normal mucosa with cancerous tissue; and 3) among different clinicopathological parameters in OSCC ( $P > 0.05$ ). The level of MICA mRNA was higher in OSCCs than in para-cancerous tissues, and was correlated with the regional lymph node status and disease stage ( $P < 0.05$ ). The levels of MICA protein and mRNA expression differ among normal oral mucosa, para-cancerous tissue, and cancerous tissue. MICA may contribute to the tumorigenesis and progression of OSCC.

**Keywords:** Carcinoma squamous cell - MHC class I-related chain A – immunohistochemistry - polymerase chain reaction

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### Introduction

MHC-I type chain-related protein A (MICA) is a newly discovered transmembrane glycoprotein encoded by MHC genes. MICA mainly functions as natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), and other immune effector cell activation receptor NKG2D ligands. These ligands can bind NKG2D-specific proteins, transmit activation signals, induce immune effector cells to clear rapidly the tumor cells of abnormal ligand expression, and play roles in immune surveillance (Bauer et al., 1999; Wu et al., 1999; Li et al., 2001).

Under normal circumstances, MICA is mainly expressed in the thymus and gastrointestinal epithelium. However, in recent years, MICA expression or overexpression has been found in numerous tumor cells, and has thus been regarded as a tumor-associated antigen (Madjd et al., 2007; Fuertes et al., 2008; Xiao et al., 2008; Zhang et al., 2008). Oral squamous cell carcinoma (OSCC) is a common malignancy of the head and neck, and knowledge on the effect of MICA expression on this cancer is limited. In the current study, we used immunohistochemistry and real-time quantitative polymerase chain reaction (PCR) to explore MICA protein and mRNA content in OSCCs. The relationship between clinical and pathological parameters was also analyzed. The aim was to increased knowledge

on OSCC immunotherapy.

### Materials and Methods

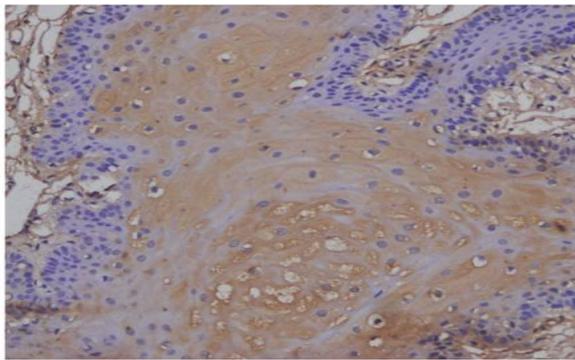
#### *Samples*

A total of 45 cases of OSCC and 14 cases of normal oral mucosa specimens were selected between February 2009 and January 2010 from the Department of Oral and Maxillofacial Surgery, Central South University Xiangya Hospital. OSCC and adjacent tissues were obtained from the tumor resection. Normal oral mucosa was obtained from non-surgical treatment of hospitalized patients without malignant tumors during the same period. All procedures were conducted in accordance with the Helsinki declaration, and with approval from the Ethics Committee of Xiangya Hospital. Written informed consent was obtained from all participants.

#### *Immunohistochemistry*

Routine paraffin sectioning, dewaxing, and hydration using 3% hydrogen peroxide were performed to remove endogenous peroxidase. Microwave antigen was retrieved and blocked with fetal calf serum for 2 h. About 50  $\mu$ l (1:25) of goat polyclonal anti-human MICA was added, and the mixture was incubated at 4 °C overnight. About 50  $\mu$ l of biotinylated goat anti-rabbit IgG secondary

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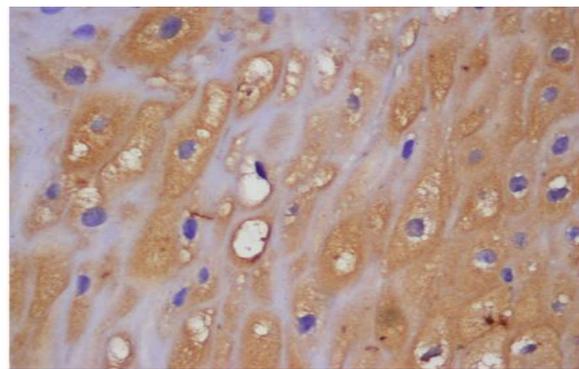
**Figure 1. MICA Expression in Normal Oral Mucosa (SP × 200)**

antibody working solution was added, and the mixture was incubated 37 °C for 30 min, followed by diaminobenzidine coloration. The sample was dyed with hematoxylin, separated using ethanol and hydrochloric acid, saturated with lithium carbonate until the color returned to blue, and then dehydrated with gradient alcohol as well as xylene. Mounting with neutral resin followed. The negative control used was PBS in place of the primary antibody. According to (Xiao et al., 2008), the staining intensity was divided into four grades based on cell counting, namely, 0 (no staining or only a nonspecific background color), 1 (light yellow), 2 (yellow), and 3 (yellow or brown). Stained cells were divided into five grades based on cell counting, namely, 0 (no staining), 1 (less than 25% stained cells), 2 (25% to 50% stained cells), 3 (50% to 75% stained cells), and 4 (more than 75% stained cells). Based on both of the above scoring methods, the immunohistochemistry results were classified into four, namely, negative (0), weakly positive (1 to 2 score), positive (3 to 4 score), and strongly positive (5 to 7 score).

#### PCR

Total RNA extraction and cDNA synthesis were performed according to kit instructions. Semi-quantitative PCR primers were designed according to MICA cDNA sequence in NCBI (GenBank No. BC016929). The upstream primer was 5'-AGCCCCACAGTCTTCGTTATA-3' and the downstream primer was 5'-CAAGGTCTGAGCTCTGGAGGA-3'. The PCR product was 412 bp long. The internal control GAPDH primer was 5'-AATCCCATCACCATCTTCCA-3' and 5'-CCTGCTTACCACCTTCTTG-3'. The PCR product was 580 bp long. For the PCR reaction, 1 µl of cDNA, 2 µl of primer, and 10 µl of 2 × PCR Master Mix were added to 20 µl of ddH<sub>2</sub>O. The reaction conditions were as follows: 95 °C denaturation for 1 min, 95 °C denaturation for 30 s, 60 °C annealing for 30 s, 68 °C extension for 90 s, 25 cycles, and 68 °C fully extended to 10 min. The PCR products were detected using 1.5% agarose gel electrophoresis, and scanned by a gel imaging analysis system camera. The band density was analyzed by the ScnImage software.

The real-time PCR primer was designed according to a previous report (Zhang et al., 2008). P1 was 5'-CCTCTGAGGCCTCCCTGCG-3' and P2 was 5'-CCTTGCCATGAACGTCAGG-3'. β-actin was used as the internal control. The primers were as



**Figure 2. MICA Located in the Cell Membrane and Cytoplasm (SP × 400)**

follows: 5'-CACTGTGTTGGCGTACAGGT-3' and 5'-TCATCACCATTGGCA ATGAG-3'. For the PCR reaction, 1 µl of cDNA, 1 µl of each primer, as well as 9 µl of a 2.5 × real Mastermix and 20 × SYBR mixture was added to 20 µl of ddH<sub>2</sub>O. The reaction conditions were as follows: 95 °C denaturation for 10 min; 95 °C denaturation for 15 s, 60 °C annealing for 60 s, 72 °C extension for 15 s, 42 cycles, 60 °C fully extended for 10 min. β-actin was used as the reference. The mean Ct was calculated using the relative quantification method (ΔΔCt) for quantitative analysis.

#### Statistical analysis

The SPSS16.0 statistical package was used for all statistical analyses. Counted data were analyzed using the non-parametric Mann-Whitney U test or Kruskal Wallis H test. Measured data were analyzed by the two paired-samples *t* test. The relationship between data was analyzed by the Spearman correlation analysis at *P* < 0.05 statistical difference significance.

## Results

#### MICA protein expression and localization

MICA was mainly expressed in the spinous layer in normal oral mucosa and para-cancerous tissues. Keratinized layer and basal expression were less (Figure 1). Expression was found in the mucosal layers in OSCC tissues. Cancer cell infiltration to the muscle tissue was also regarded as positive expression.

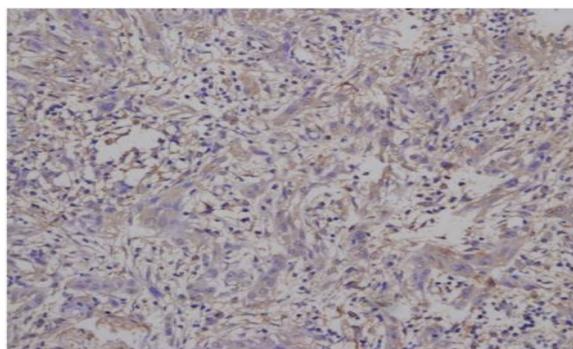
Under high magnification, MICA was expressed as light yellow or brown in the cytoplasm and cell membrane (Figure 2). The expression intensity followed the trend para-cancerous tissue > normal oral mucosa > OSCC tissue (Figures 3 and 4). The expression intensity was significantly higher in para-cancerous tissue than in OSCC tissue (*Z* = -2.099, *P* = 0.036). However, no statistically significant difference was found between para-cancerous tissue and normal oral mucosa (*Z* = -0.192, *P* = 0.848), as well as between normal mucosa and OSCC tissue (*Z* = -1.388, *P* = 0.165). There was also no statistically significant difference among the different clinical and pathological parameters of OSCCs of the different groups (*P* > 0.05) (Table 1).

#### MICA mRNA expression

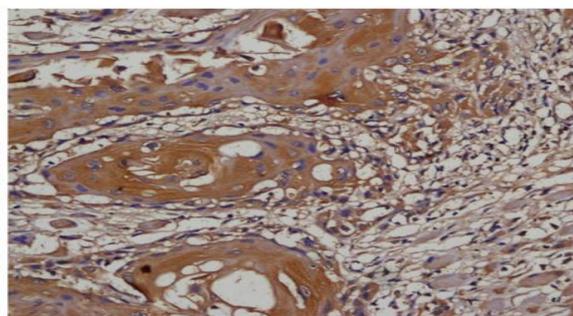
Among the 45 cases of OSCC and adjacent tissues,

**Table 1. Comparison of MICA Protein and mRNA Expression in Different Clinical and Pathological Parameters of Oral Squamous Cell Carcinoma**

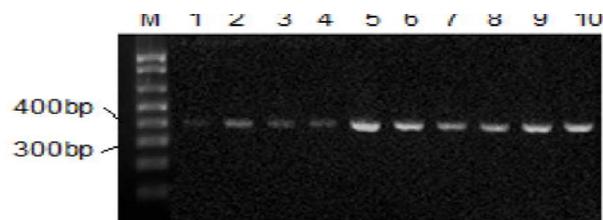
Clinicopathological parameter		Case	MICA protein expression(case)					MICA mRNA expression					
			-	+	++	+++	X <sup>2</sup> value	P value	case	ΔCt value	t value	P value	
Gender	Male	36	3	5	20	8	-1.445	0.148	20	8.52±1.50	0.729	0.472	
	Female	9	2	1	6	0			9	8.12±0.93			
Age	<50	19	2	3	12	2	-0.733	0.464	13	8.31±1.22	-0.315	0.755	
	≥50	26	3	3	14	6			16	8.47±1.48			
Tumor size	T1+T2	35	5	5	19	6	-1.008	0.313	24	8.30±1.42	-0.811	0.424	
	T3+T4	10	0	1	7	2			5	8.84±0.91			
Clinical stage	I+II	29	5	3	17	4	-1.168	0.243	19	7.94±1.27	-2.826	0.009	
	III+IV	16	0	3	9	4			10	9.27±1.05			
Tumor differentiation	High	35	5	3	20	7	-0.489	0.625	22	8.52±1.49	0.871	0.391	
	Middle	10	0	3	6	1			7	8.01±0.67			
Cervical lymph node metastasis	No	33	5	4	20	4	-1.551	0.121	20	7.91±1.25	3.451	0.002	
	Yes	12	0	2	6	4			9	9.49±0.83			



**Figure 3. Oral Squamous Cell Carcinoma of MICA Weakly Positive (SP × 200)**



**Figure 4. MICA Positive Expression Strongly in Oral Squamous Cell Carcinoma (SP × 200)**



**Figure 5. MICA mRNA Gel Electrophoresis in Oral Squamous Cell Carcinoma and Adjacent Noncancerous Tissue. 1-4) adjacent tissue; 5-10) cancer tissue**

16 cases were removed due to the poor quality of RNA extraction or degradation; 29 cases were used performed. The relative MICA mRNA expression of OSCC ( $8.40 \pm 1.34$ ) was significantly higher than that in adjacent tissue ( $6.50 \pm 2.46$ ) ( $t = -4.015$ ,  $P = 0.000$ ), consistent with the semi-quantitative PCR results (Figure 5). Different clinical stages and neck lymph node metastasis between groups

were significantly different ( $P < 0.05$ ) (Table 1).

*Correlation analysis between MICA protein and mRNA expression*

There was no correlation between MICA protein and mRNA expression in OSCC tissues ( $r = 0.085$ ,  $P = 0.707$ ).

**Discussion**

NK and CTL cells are the main antitumor immune effector cells of the body. Their functions are closely related to cell surface C-type lectin-like NKG2D receptor activation (Bauer et al., 1999). There are three main NKG2D ligands identified in humans, namely, MICA, MICB, and UL16-binding protein. However, studies indicate that MICA plays a major role in antitumor immunity (Sutherland et al., 2002; Jinushi et al., 2003).

MICA encoded by MHC genes includes 383 amino acids with a molecular weight of 43 kDa. In recent years, studies have found that MICA is expressed or overexpressed in numerous tumor cells, such as breast, lung, ovarian, colon, kidney, pancreatic, prostate cancer, hepatocellular carcinoma, and melanoma. Thus, MICA is also regarded as a tumor-associated antigen.

MICA is mainly expressed in the thymus and gastrointestinal epithelium under normal circumstances. The current study revealed that MICA was mainly expressed in the spinous layer of normal oral mucosa. Given that the oral mucosa and gastrointestinal tract mucosa belong to the gastrointestinal epithelium, our findings are consistent with previous results (Ostberg et al., 2007).

The real-time PCR results suggest that MICA mRNA expression in OSCC tissue was significantly higher than in adjacent tissues, and was positively correlated with the cervical lymph node metastasis and clinical stage. These findings were consistent with those of Liu et al. (2007). However, the immunohistochemical results demonstrated that MICA protein expression in OSCC tissue was lower than in adjacent tissues, and the difference was not statistically significant between clinical pathological parameters. Further correlation analysis also confirmed the absence of correlation between MICA protein and mRNA expression in OSCC tissue.

MICA protein and mRNA expression levels are

inconsistent probably because of the existence of different post-translational processes and/or transport pathways. In some epithelial cell lines, although the mRNA expression levels are similar, MICA expression on the cell surface is very low or even undetectable. A recent study has indicated that tumor cell surface membrane-bound MICA could be converted into the free state of soluble MICA (sMICA) via a variety of mechanisms. sMICA can combine with NKG2D to induce NKG2D self-degradation and self-digestion. Consequently, the activation of signal transduction becomes limited, thereby contributing to tumor escape immune response (Kaiser et al., 2007; Waldhauer et al., 2008).

A previous study (data not shown) has also demonstrated that serum sMICA expression levels in patients with OSCC tissue were higher than in normal ones. This result is related to the tumor size, clinical stage, and lymph node metastasis. In the current study, compared with adjacent tissues, MICA mRNA in OSCC tissue was higher, but the protein content was lower. This finding can be attributed to the fact that MICA on the surface of tumor cells shed more compared with adjacent tissue. However, the specific mechanism for this difference still needs further study (Tamaki et al., 2008).

MICA protein and mRNA expression in normal oral mucosa, OSCC tissue, and adjacent tissues were different, suggesting the involvement of MICA in the development and progression of OSCC. The inconsistency between MICA protein and mRNA expression levels may pave a new pathway for OSCC immunotherapy. If MICA shedding could be blocked from tumor cells, the tumor cell surface expression of MICA would increase to induce rapidly the removal of tumor cells by CTL and NK cells. The concentration of serum sMICA is also decreased to prevent NKG2D self-digestion and self-degradation, which is very beneficial to the improvement of the antitumor immunity of a patient.

## Discussion

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