J Oral Pathol Med (2007) 36: 43–7

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MICA and *MICB* overexpression in oral squamous cell carcinoma

Chung-Ji Liu[1,2,3], Man-Tin Lui[1,4], Hsin-Lan Chen[1], Shu-Chun Lin[1], Kuo-Wei Chang[1,4]

[1]*School of Dentistry, National Yang-Ming University, Taipei;* [2]*Department of Oral and Maxillofacial Surgery, Mackay Memorial Hospital, Taipei;* [3]*Mackay Medicine, Nursing, Management College, Taipei;* [4]*Department of Oral and Maxillofacial Surgery, Taipei Veterans General Hospital, Taipei, Taiwan*

BACKGROUND: The concentration of MICA in serum seems be a good candidate marker in cancer. Previous studies from our laboratory have shown that the polymorphic *MIC* gene may confer a risk for oral squamous cell carcinoma (OSCC). The study investigated the expression levels of *MICA* and *MICB* of OSCC patients and cancer cell lines.

MATERIAL AND METHODS: We used RT-PCR to analyze the mRNA expression of *MICA* and *MICB* in four oral cancer cell lines compared with three normal human oral keratinocyte (NHOK) cell lines and in tissues from 36 patients with OSCC comparing tumor tissue with non-cancerous matched tissue (NCMT).

RESULTS: Endogenous *MICB* mRNA expression in OSCC cell lines was significantly higher than that in NHOK (1.40 ± 0.27 vs. 0.40 ± 0.16; $P = 0.04$). In 20 of 36 sets of tissue from patients with OSCC, *MICB* mRNA expression was higher in the cancerous tissue than in the NCMT. The mean *MICB* mRNA expression in OSCC tissues was significantly higher than in NCMT (0.39 ± 0.08 vs. 0.14 ± 0.03, $P = 0.009$, paired *t*-test). A significantly lower *MICA* mRNA was found in patients who chewed areca nut compared with those who did not use areca ($P = 0.001$) and in patients with well-differentiated tumors compared with those with less well-differentiated tumors ($P = 0.02$).

CONCLUSION: *MICA* and *MICB* mRNA expression may be increased in OSCC but there appears to be individual variation.

J Oral Pathol Med (2007) **36**: 43–7

Keywords: MICA; MICB; mouth neoplasms; RT-PCR

Correspondence: Dr Kuo-Wei Chang, School of Dentistry, National Yang-Ming University, Sec. 2, No. 155, Li-Nong St, Peitou, Taipei 112, Taiwan. Tel: +8862 28267223, Fax: +8862 28264053, E-mail: ckcw@ym.edu.tw
Accepted for publication June 6, 2006

Introduction

Oral squamous cell carcinoma (OSCC) is not a single gene disorder but rather the result of a series of gene mutations (1, 2). In addition to oncogenes and tumor suppressor genes, a variety of immune mediators and external factors also play important roles in OSCC (3, 4). Tumorigenesis is a sequential process involving multiple gene mutations that affect cell cycle and proliferation.

Alterations of immune function have been detected in patients with OSCC (5–8). A major step in oncogenesis is the evasion by tumor cells of immune surveillance as well as production of immunosuppressive cytokines (9, 10). For this reason, tumor immunity is increasingly a target of research. The most important genetic component of the body's immune function is the major histocompatibility complex, and certain human leukocyte antigens (HLA) have been implicated in the development of squamous cell carcinoma (11–13), including both HLA class I and class II genotypes (14). Loss of heterozygosity of the HLA complex may provide tumor cells with a phenotype permitting immune escape (15). HLA class I expression in OSCC regulates natural killer (NK) cell activity (16).

In addition to the classical class I peptide-presenting molecules, a new polymorphic gene family, the *MIC* gene (MHC class I chain-related gene), has been described (17). *MICC*, *MICD*, and *MICE* are in fact pseudogenes, but *MICA* and *MICB* encode mRNA under conditions of biological stress such as infection or cancer (18, 19). These two genes have 84% homology (20, 21). Although *MICB* is not expressed under normal conditions, it is highly expressed in epithelial tumor cells in the presence of infection with bacteria or viruses. The resulting *MICB* protein product directly interacts with T cells and is therefore important in maintaining the normal function of oral epithelial cells (22, 23).

During the development of cancer, the expression of *MIC* genes may play an important role by becoming antigens against tumor cells. NKG2D is a C-type

lectin-like active immunoreceptor (18, 24) expressed by NK, CD8αβ T, and γδ T cells (25, 26). γδ T cells are T cells found in the submucosa. Human NKG2D binds to *MICA* and *MICB* (26) as well as to molecules belonging to the UL-16-protein (ULBP) family. In addition, NKG2D ligands are expressed on many tumor cells (27). Based on these findings, the NKG2D receptor/ligand system has been interpreted as a sensor system specifically for the recognition of 'stressed' cells and tumors (28). The expression level of the MIC may make the tumor cells to be interpreted via the MIC-NKG2D systems (28).

Previous studies seem to indicate that the concentration of MICA in the blood is an indicator for cancer (28). The *MICA* gene polymorphism was associated with OSCC in our previous study (23). However, the function of MICA and MICB in OSCC is not known. We designed this study to investigate the expression of *MICA* and *MICB* in normal human oral keratinocytes (NHOK) and OSCC.

Materials and methods

Patients and cells

This study was approved by our Institutional Review Board. NHOK were obtained from three patients undergoing a flap operation for removal of impacted wisdom teeth. The cells were cultured in KSFM (Life Tech., Gaithersburg, MD, USA) (6). OEC-M1, OC-3, SAS and SCC25 OSCC cell lines were cultured following standard protocols (6, 29).

Surgical specimens were obtained from 36 patients with OSCC treated in the Oral and Maxillofacial Department of MacKay Memorial Hospital, including both tumor and non-cancerous matched tissue (NCMT). The age of the patients ranged from 29 to 76 years with a mean of 49 years. The most common primary site was the buccal mucosa (50%, 18 cases). Five of thirty-six (14%) were well differentiated and 31 (86%) were moderately or poorly differentiated. Nineteen (53%) of the patients had lymph node metastases, and 21 (58%) had stage IV disease (Table 2). To insure that there was no contamination by tumor cells in the sample and to avoid prejudicing the evaluation of the surgical limit, NCMT was samples 1 cm away from the resection margin. No dysplasia or neoplasia was noted in the NCMT. All the tumors exhibited infiltrating characteristics and contained more than 70% neoplastic cells. Whole tissues were prepared for RNA extraction.

RT-PCR

Total RNA isolation and reverse transcription reaction were performed according to previously described protocols (6). The sense and antisense primers for MICA were 5′-ATGGGGCTGGGCCCGGTCTTC-3′ and 5′-AGCAGAAACATGGAATGTCTGCCAA-3′. The size of the PCR product was 936 bp (30). The sense and antisense primers for MICB were 5′-ATGGG GCT GGGCCGGGTCCTGCTGTTT-3′ and 5′-AGAA ACATATGGAAAGTCTGTCCGT-3′, and the size of the PCR product was 915 bp (30). Amplification of a 190-bp glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) amplicon was used as an internal control. After electrophoresis, the densities of the amplicons were measured by a densitometer (Amersham, Piscataway,

Table 2 Clinical and tumor variables and *MICA* and *MICB* mRNA expression

	MICA		*MICB*	
	OSCC > NCMT	*OSCC ≤ NCMT*	*OSCC > NCMT*	*OSCC ≤ NCMT*
Age				
> 52	5	11	10	7
≤52	10	7	10	6
P	0.17		1.00	
Areca				
Yes	8	18	15	11
No	7	0	5	2
P	0.001**		0.68	
Site				
BM	5	9	10	4
Non-BM	10	9	10	9
P	0.48		0.31	
Differentiation				
Moderate	15	12	15	12
Well	0	6	5	1
P	0.02*		0.36	
Stage				
I–III	8	8	11	5
IV	7	10	9	8
P	0.73		0.48	
LNM				
T1–T3	9	10	13	6
T4	6	8	7	7
P	1.00		0.47	
LNM				
$n = 0$	6	11	8	9
$n \geq 1$	9	7	12	4
P	0.30		0.16	

OSCC, oral squamous cell carcinoma; NCMT, non-cancerous matched tissue; BM, buccal mucosa, LNM, lymph node metastasis. Fisher's exact test.

Table 1 *MICA* and *MICB* mRNA expression in NHOK and OSCC cells

	NHOK (n = 3)	*OSCC (n = 4)*	*NCMT*	*OSCC*
MICA mRNA	1.38 ± 0.47	2.26 ± 0.23	0.88 ± 0.17	1.08 ± 0.15
P		0.12		0.15
MICB mRNA	0.49 ± 0.16	1.40 ± 0.27	0.14 ± 0.03	0.39 ± 0.03
P		**0.04***		**0.009****

Non-paired *t*-test. NHOK, normal human oral keratinocyte; OSCC, oral squamous cell carcinoma; NCMT, non-cancerous matched tissue.
*: significant; **: very significant.

NJ, USA). All experiments were repeated three times. The ratios of *MICA* and *MICB* mRNA expression were corrected by the ratio of *G3PDH*. An OSCC/NCMT mRNA expression ratio of >1.2 was defined as increased mRNA expression, that is, as gene overexpression.

Statistical analysis

A paired *t*-test or Fisher's exact test was used to analyze the mRNA expression ratios of *MICA* and *MICB* in OSCC and NCMT. Data are reported as mean ± standard deviation. Survival analysis was performed using the Kaplan–Meier method. $P < 0.05$ was considered as statistically significant.

Results

Expression of MICA *and* MICB *in cell lines*

We compared the mRNA expression of *MICA* and *MICB* in three NHOK lines and the four OSCC cell lines (Fig. 1). The expressions of both genes were higher in the OSCC lines than in NHOK (Fig. 2), but the difference was significant only for *MICB* ($P = 0.04$) (Table 1).

mRNA expression of MICA *and* MICB *in OSCC*

A similar result was found when we compared *MICA* and *MICB* mRNA expression in OSCC and their NCMTs. The mean *MICA* expression was 0.88 ± 0.17 in NCMT vs. 1.08 ± 0.15 in OSCC tissue ($P = 0.15$) and that of *MICB* 0.14 ± 0.03 vs. 0.39 ± 0.08 ($P = 0.009$) (Figs 3 and 4). Thus only *MICB* was significantly overexpressed in OSCC tissues. The mRNA expressions of *MICA* and *MICB* did not correlate with one another ($P = 0.17$, $R^2 = 0.06$).

The seven patients who had no history of chewing areca had significantly higher *MICA* mRNA than those who did use areca ($P = 0.001$). By contrast, *MICA* mRNA was not increased in patients with well-differentiated tumors, a significantly different result than in those with less well-differentiated tumors ($P = 0.02$). Although the mRNA expression of *MICB* was increased in 20 out of the 36 patients with OSCC, it did not correlate with age, disease site, lymph node metastasis, or cancer stages (Table 2).

Patients with overexpression of either *MICA* or *MICB* had slightly poorer survival than patients without

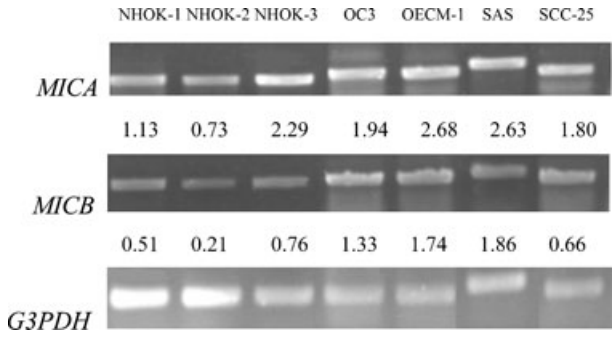

Figure 1 Endogenetic *MICA* and *MICB* mRNA expression by RT-PCT in three normal human oral keratinocyte (NHOK) lines and four oral squamous cell carcinoma (OSCC) cell lines. NHOK-1 was from a 20-year-old female, NHOK-2 from a 21-year-old female, and NHOK-3 from a 16-year-old male. For each line, the third generation of cells was used.

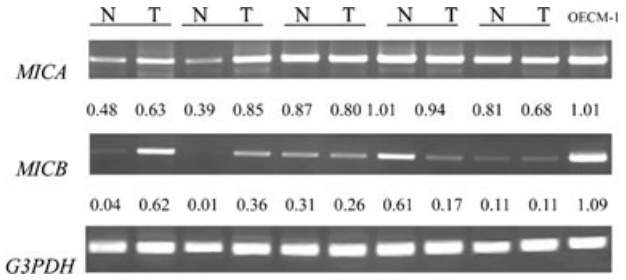

Figure 3 *MICA* and *MICB* mRNA expression in oral squamous cell carcinoma (OSCC) and non-cancerous matched tissue (NCMT; $n = 36$) by RT-PCR (five representative results). *G3PDH* was the internal control and the OEC-M1 cell line was the positive control. N: NCMT; T: OSCC tissue.

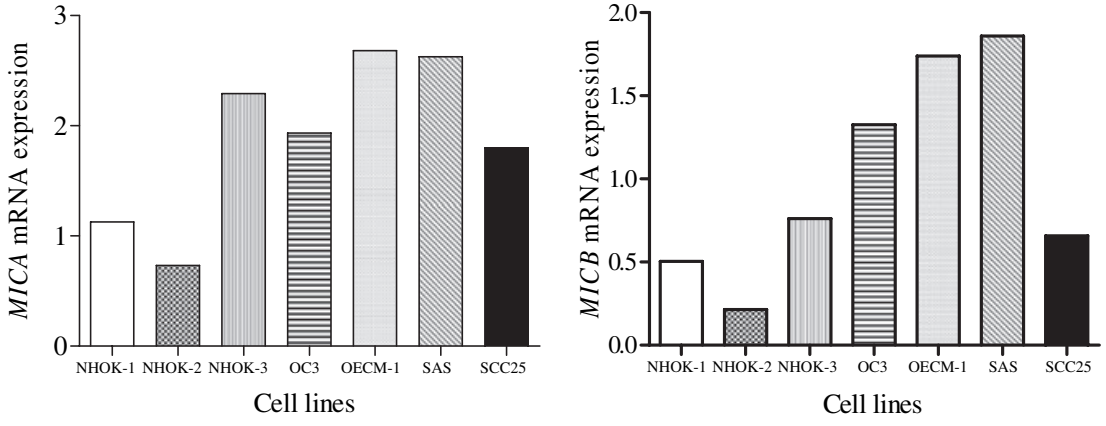

Figure 2 Glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*)-corrected *MICA* and *MICB* expression in three normal human oral keratinocyte (NHOK) lines and four oral squamous cell carcinoma (OSCC) cell lines.

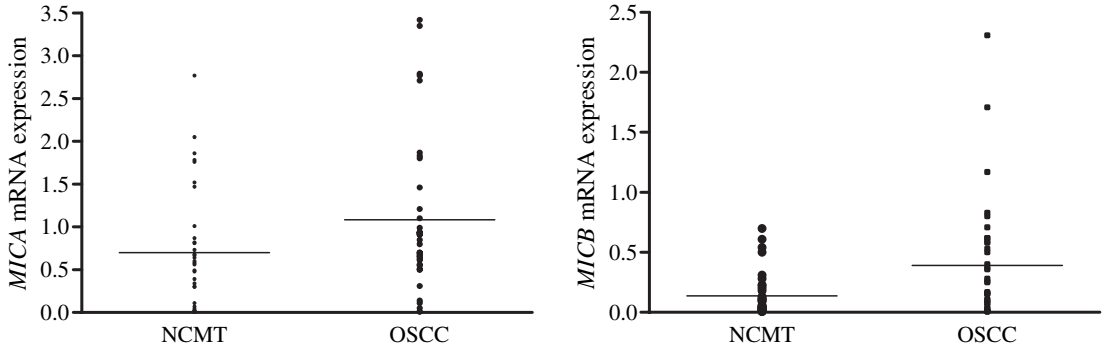

Figure 4 *MICA* and *MICB* mRNA expression for oral squamous cell carcinoma (OSCC) vs. non-cancerous matched tissue (NCMT; $n = 36$) corrected by *G3PDH* and compared with a paired *t*-test. *MICA*: 1.08 ± 0.15 vs. 0.88 ± 0.17, $P = 0.15$; *MICB*: 0.39 ± 0.08 vs. 0.14 ± 0.03, $P = 0.009$.

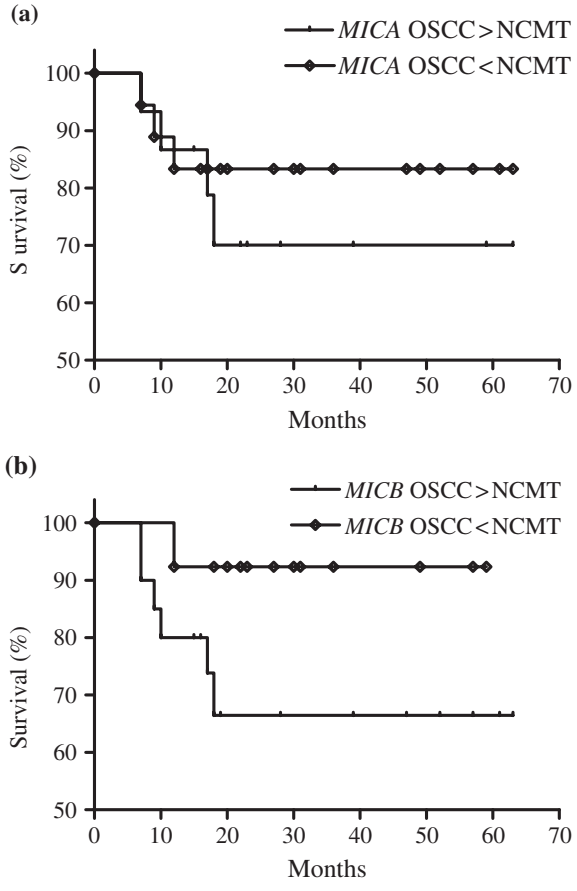

Figure 5 Survival curves for patients with and without overexpression of *MICA* (A, $P = 0.49$) and *MICB* (B, $P = 0.10$).

the corresponding overexpression, but the differences were not significant (Fig. 5).

Discussion

In this study, we used a variety of OSCC cell lines (OC3 and OECM-1 from Taiwan and SCC25 and SAS from the USA and Japan) and found that all had an increased mean expression of *MICA* and *MICB* compared with the mean expression by NHOK, although the only statistical difference was in *MICB* expression. Both genes are expressed in NHOK, although it is unclear whether this represents constitutive expression. The mRNA expression of *MICA* and *MICB* in one of the three NHOK lines we studied was actually higher than the two cancer cell lines. NHOK-3 was from a 16-year-old male. It may be caused by the individual and age variations. Generally, the expression of *MICA* and *MICB* differs according to tissue site.

Jinushi et al. investigated *MICA* and *MICB* expression in 10 liver cancer specimens paired with normal tissue (31). Both genes were expressed in most of the cancer specimens, and they were overexpressed in 5 out of the 10 specimens, a similar proportion to the 18 out of 36 specimens in our study that expressed these genes, although only *MICB* was significantly overexpressed. Bahram and Spies (18) stated that, if the pressure is changed, the relative expression of *MICB* is less than that of *MICA* mRNA. But in our experiments, the mean *MICA* expression was higher than that of *MICB* mRNA. This discrepancy may relate to variation in amplification by PCR. Further testing with Northern blot analysis is needed. On linear regression, we found no correlation between the expression of the two genes, suggesting they are not competitive, at least in terms of mRNA production. It may be that different tumors have different levels of expression of the genes. Assays for the protein in OSCC might clarify this issue.

In our evaluation of clinical and tumor characteristics, we found that overexpression was slightly but not significantly associated with decreased survival. Long-term follow-up for these cases may help clarify the decreased tendency of gene overexpression. Interestingly, patients who chewed areca were less likely to have *MICA* overexpression than those who did not chew it. Areca is known to be a risk factor for OCSS (32–34). Our results suggest that the mechanism by which it contributes to carcinogenesis does not involve *MICA*. On the other hand, patients with moderately to poorly differentiated cancers were more likely to have *MICA* overexpression than those with well-differentiated tumors. This suggests that *MICA* might be involved in the loss of differentiation in OSCC tissues. Other variables including age, tumor site, lymph node metastasis, and

tumor stage were not significantly correlated with the expression of either *MICA* or *MICB*. Studies involving larger series of patients and assessment of the *MIC* protein product would be helpful in further assessing important clinical correlations.

In summary, we have shown that both *MICA* and *MICB* are expressed in OSCC tissues, suggesting that the products of these genes may contribute to carcinogenesis.

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Acknowledgements

The authors thank Dr M.J. Buttrey for her kind review and instructive criticism of this paper. This study is supported by Grant NSC-93-2314-B-195-021 from the National Science Council, Taipei, Taiwan.