Soluble MICB Serum Levels Correlate with Disease Stage and Survival Rate in Patients with Oral Squamous Cell Carcinoma

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Abstract. *Background: Expression of ligands of natural killer group 2D (NKG2D) immunoreceptors, such as major histocompatibility complex class I-related chain A/B (MICA/B), has been proposed to play an important role in tumour immunosurveillance. Soluble forms of MICA/B are increased in sera of cancer patients and are postulated to impair antitumour immune response by downregulating expression of NKG2D immunoreceptors. Serum levels of soluble MICA have been shown to be of diagnostic significance in malignant diseases. Aims: The potential of soluble MICB (sMICB) as a marker for oral squamous cell carcinoma (OSCC) was investigated. Results: sMICB levels did not differ significantly from those in normal control individuals. However, the findings indicate that sMICB levels are significantly increased in stage IV OSCC and high sMICB levels are significantly associated with decreased survival rates in patients.*

Oral squamous cell carcinoma (OSCC) is a solid tumour of epithelial origin. It is the sixth most common cancer globally, although its incidence varies dramatically with location. The relative frequency of OSCC ranges from less than 1% to over 40% amongst all malignancies (1). Approximately 6,000 cases of OSCC are diagnosed annually in Japan, and it is related to tobacco and alcohol consumption (2).

Despite aggressive and often mutilating therapeutic regimens, overall survival in OSCC has remained largely unchanged over the past 20 years. Improvement in long-term survival rate of patients with OSCC requires identification of prognostic markers that can differentiate patients with a high risk for local recurrence and lymph node metastasis.

The human leukocyte antigen (HLA) system plays an important role in the cellular immune response to viral and tumour antigens (3). The HLA region is located on chromosome 6p21.3 and encompasses a 4 Mb segment that has evolved through repeated gene duplication and conversion events (4).

The major histocompatibility complex (MHC) class I chain-related (MIC) gene family, which lies within the HLA region, consists of five members: *MICA, MICB, MICC, MICD,* and *MICE*. MICA and MICB (generally termed MIC) are the most investigated natural killer group 2D (NKG2D) ligands which have been proposed to mediate tumour rejection (5, 6). MIC protein can trigger natural killer (NK) cell activity against cancers *via* the NKG2D and DNAX-activating protein (DAP) 10 or DAP12 complex expressed on many immune cell surfaces (7).

MIC are MHC class I homologues that play no role in antigen presentation. MICA, along with UL16-binding proteins (ULBPs), act as ligands for the immunostimulatory C-type lectin-like receptor NKG2D, which is expressed on most NK cells, CD8 positive (CD8^{+}) T-cells, and gamma delta ($\gamma\delta$) T-cells (8, 9).

MIC expression is normally restricted to areas of the intestinal epithelium with limited surface expression. Under pathological conditions, such as cancer, infection, hypoxia, heat shock, and oxidative stress, MIC expression is induced (9).

Several studies have described a possible mechanism for tumour escape from NKG2D-mediated immunosurveillance in humans. Namely, tumour cells reduce NKG2D ligand surface levels by shedding MIC in a soluble form. The soluble form engages cells expressing NKG2D, inducing endocytosis and degradation of this receptor (10).

Soluble MIC released by epithelial tumours down-regulates NKG2D surface expression and leads to a functional impairment of tumour antigen-specific cytotoxic lympho-

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Table I. *Clinical parameters of 60 patients with OSCC.*

Parameter	Frequency (%)
Age, years, mean (range)	67.15 (38-91)
Gender (male/female)	34/26
Tumour size	
T1	18 (30.0)
T2	17 (28.4)
T3	15 (25.0)
T4	10 (16.6)
Disease stage	
I	18 (30.0)
II	17 (28.4)
III	12 (20.0)
IV	13 (21.6)

Table II. *Clinical parameters of 60 patients with OSCC.*

Parameter	Frequency (%)
Tumour differentiation	
Well	27 (45.0)
Moderate	23 (38.4)
Poor	10 (16.6)
Tumour location	
Tongue	39 (65.0)
Gingiva	17 (28.3)
Bucca mucosa	4 (6.7)

Table III. *Levels of soluble MICB and clinical parameters of patients with OSCC.*

Parameter	Soluble MICB, mean (range) (pg/ml)
Healthy controls	21.2±16.1 (0-46)
All patients	
Tumour size	
T1	12.4±16.2 (1-45)
T2	21.7±10.5 (0-98)
T3	31.1±13.7 (9-97)
T4	30.6±16.2 (5-77)
Tumour differentiation	
Well	24.1±17.4 (0-98)
Moderate	24.3±14.5 (5-97)
Poor	27.0-10.1 (5-75)
Disease stage	
I	19.7±11.7 (1-45)
II	22.0±14.6 (0-98)
III	30.1±17.2 (6-76)
IV	37.8±10.1 (5-97)

In this study, the expression of sMICB in patients with OSCC was investigated. MICB-specific sandwich enzyme-linked immunosorbent assay (ELISA) was implemented to investigate sMICB release by tumour cells and sMICB levels in OSCC patients. sMICB was correlated with tumour stage to evaluate the potential diagnostic power of combining both markers.

Materials and Methods

Study patients. Sixty patients (34 males, mean age: 69.0 years; 26 females, mean age: 69.2 years) with OSCC and 50 unrelated healthy control individuals (22 males, mean age: 35.1 years, 28 females, mean age: 39.9 years) participated in this study. The patients were seen at the Department of Oral and Maxillofacial Surgery in Nara University Hospital between May 2003 and December 2007. The Ethics Committee of Nara Medical University approved this study.

Serum samples were obtained from all patients prior to surgery, which was the most frequent treatment modality, or before the start of chemo- or radiotherapy. Peripheral blood was collected after obtaining informed consent. Clinical data (age, gender, tumour location, tumour size, grade of tumour differentiation, and disease stage) were extracted from medical records (Table I and II). Primary tumour size, lymph node status, and disease stage were classified according to the 1997 TNM criteria (21).

ELISA. Serum levels of soluble MICB have been previously determined using sandwich ELISA (16, 17). In brief, plates were coated with the capture anti-MICB monoclonal antibody (mAb) AMO-1 at 2 μg/ml in PBS, then blocked with 100 μl of 15% BSA for 2 h at 37°C and washed. Next, the standard and the samples were added and plates were incubated for 2 h at 37°C. Sera were diluted 1:10 in 5% BSA before being added to the plates. After incubation, plates were washed and incubated with the detection mAb BAMO-3 (5 μg/ml in 7.5% BSA-PBS) for 2 h at 37°C. Plates
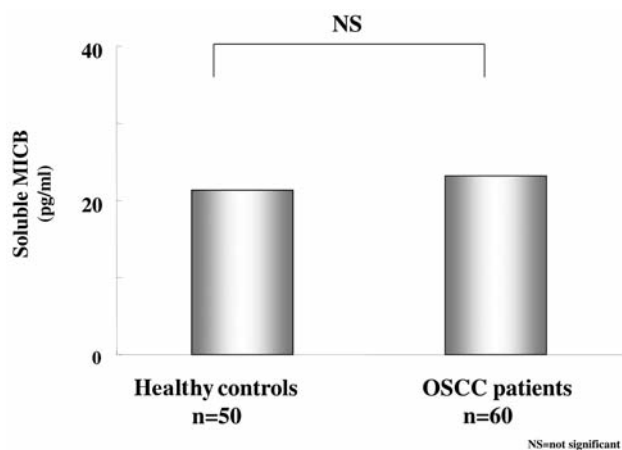
cytes (11). Therefore, MIC may function as a self-antigen that can be stress-induced and recognized by T-cells endowed with the TCR variable region Vδ1, which represent 70-90% of γδ T-cells in the intestinal epithelium (11, 12). These results suggest that soluble MIC may play a critical role in tumour immune evasion. They also raise the question of whether soluble MIC may be of diagnostic value in cancer patients. Soluble MICA (sMICA) has thus far been detected in sera of patients with prostate and colorectal, pancreatic cancer, and several other malignancies (13-15). Recently, it was reported that soluble MICB (sMICB) may impair tumour immunogenicity by reducing NKG2D ligand densities on malignant cells (16-18). Moreover, presence of sMICA molecules was also described in sera of heart transplant individuals and was associated with lower incidence of rejection (19).

It has previously been demonstrated that sMICA levels are elevated in OSCC patients compared with healthy controls and significantly correlated with stage IV disease (20). However, it is not known whether sMICB can be similarly used as a tumour marker or add to the diagnostic value of sMICA.

Figure 1. *Levels of soluble MICB in sera of OSCC patients and healthy controls.*
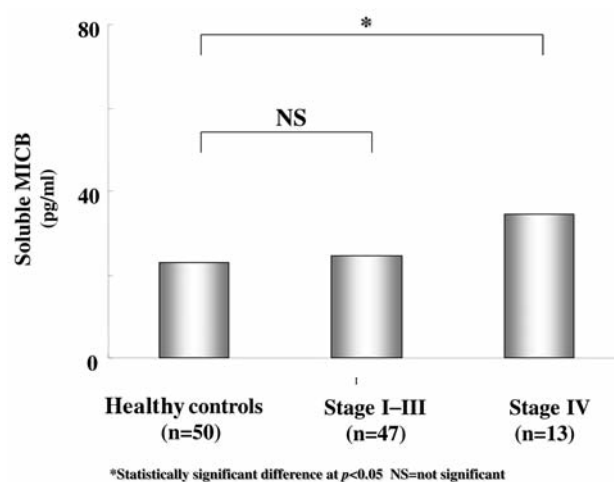

Figure 2. *Levels of soluble MICB in sera from healthy controls and OSCC patients stratified by disease stage.*

were then washed and incubated with anti-mouse IgG2a-HRP (1:8000 in 7.5% BSA-PBS) for 1 h at 37°C (R&D Systems, MN, USA). Plates were washed again and developed using the tetramethylbenzidine peroxidase substrate system (R&D Systems, MN, USA). Absorbance was measured at 450 nm. Soluble MICB levels greater than 5.0 pg/ml were considered positive based on the detection limit of the ELISA; this level corresponded to 15.0 pg/ml in patient serum.

Statistical analysis. The difference in distribution of soluble MICB levels between OSCC patients and healthy controls was statistically tested using nonparametrical analysis, the paired *t*-test, and Fisher's exact test. A statistically significant difference was defined as $p < 0.05$.

Overall and progression-free survival analyses were performed using Kaplan-Meier curves. Statistical significance of differences in disease-specific survival between groups with different sMICB levels was estimated using the log-rank test.

All patients whose deaths were associated with OSCC, including those dying from postoperative complications, were included in disease-specific survival calculations.

Results

Levels of soluble MICB in sera of OSCC patients were compared to those from unrelated healthy control individuals (Table III). While control sera demonstrated low sMICB levels close to the detection limit of the ELISA, patients possessed higher sMICB levels on average. However, this difference became non-significant after using corrected *p*-values (Figure 1) ($p=0.078$).

It was then assessed whether stratification of patients with respect to pathological parameters such as tumour size, grade of tumour differentiation, and disease stage would reveal associations with sMICB levels (Table III). Tumour size and differentiation were not associated with sMICB levels.

However, there was a trend for higher sMICB levels in stage IV OCSS patients (Figure 2) ($p=0.045$)

A cut-off point was established using a receiver operating characteristic (ROC) curve to distinguish between high MICB (>24 pg/ml, N=23) levels, and low MICB (<24 pg/ml, N=37) levels. Kaplan-Meier analysis and log-rank test revealed overall and progression-free survival of OSCC patients. High sMICB (N=12) levels were significantly associated with decreased survival rates in patients (Figures 3 and 4).

Discussion

Recent studies have highlighted the significance of NKG2D and MIC function in T-cell-mediated tumour immunity. However, the role of the NKG2D ligands MIC and ULBP2 in the biology of malignancies remains poorly understood.

MIC and other NKG2D ligands are broadly expressed on various tumours. Tumour cells have been demonstrated to release MIC in soluble form by metalloprotease-mediated proteolytic shedding.

Elevated levels of sMICA can be detected in sera of patients with prostate cancer, colon adenocarcinoma and pancreatic cancer (13-15). Increases in sMICA in advanced stages of some tumours have been reported (23). However, little is known about sMICB in OSCC patients. In the present study, it was demonstrated that sMICB increased in stage IV OSCC. To the Authors' knowledge, this is the first study to determine the significance of sMICB in OSCC patients.

Expression of MIC is associated with tumour transformation. Specifically, cell surface MICA has been observed in epithelial malignancies including lung, gastric, renal, colon, ovarian, pancreatic, and oral tumours (13-15, 22). In one study using a mouse model, tumour cells
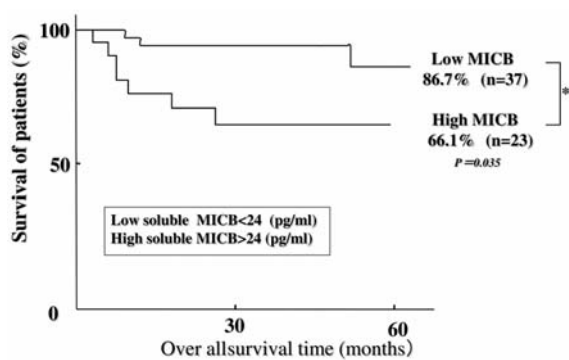
Figure 3. *Kaplan-Meier plot for OSCC patients showing survival according to levels of soluble MICB.*
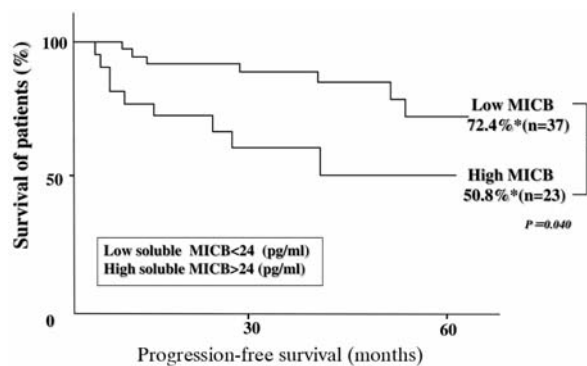

Figure 4. *Kaplan-Meier plot for OSCC patients showing progression-free survival according to levels of soluble MICB.*

expressing high levels of NKG2D ligands were targeted by NK cells, CD8^{+} T-cells, and γδ T-cells for eradication.

The exact mechanism by which soluble MIC reduces tumour immunogenicity is unclear. Proteolytic cleavage of MIC depletes the amount of NKG2D ligand on tumour cell surfaces, resulting in a weakening of the anti-tumour immune response by innate cells. Upon binding of the soluble ligand to NKG2D on NK cell surfaces, the complex is internalized and cell cytotoxic function is reduced (22-24).

Whether a tumour evades immune surveillance or becomes eradicated is dependent on its recognition by innate immune cells. Tumour-specific ligands that activate or inhibit immune cells may therefore be viable molecular markers of tumour prognosis. For example, one study of 23 prostate cancer patients found an association between serum sMICA levels and disease progression (13). In a study of 512 cancer patients, elevated sMICA and sMICB serum levels correlated significantly with cancer stage and metastasis (22). However, the ability to use sMICA levels to accurately diagnose stage and aggressiveness of all malignancies remains to be determined.

A recent report provided evidence that serum TGF-β1 in lung and colorectal cancer patients impairs NK cell activity *via* NKG2D downregulation (12). Gene silencing of TGF-β1 and TGF-β2 revealed that malignant glioma cells do not down-regulate NKG2D expression in a NK cell line. Moreover, strong surface MIC expression on tumour cells was also observed with blockage of TGF-β production, promoting a strong recognition by immune cells (23-25). These results support a key role of TGF-β in the MIC/NKG2D pathway.

Several reports showed that soluble MIC levels were similar between cancer patients and controls, suggesting that soluble MIC was not responsible for down-regulating NKG2D on CD8^{+} T-cells in these cancer patients. However, transwell experiments demonstrated that direct contact between CD8^{+} T-cells and MIC-expressing tumour cells caused NKG2D down-regulation.

Recent work has shown that both MIC and ULBP2 on the cell membrane may be cleaved by metalloproteases to produce soluble molecules. These soluble forms down-regulate surface expression of NKG2D receptors, thereby impairing the anti-tumour reactivity of NK and CD8^{+} T-cells (23-25).

OSCC patients with high sMICB levels had significantly lower survival rates. Furthermore, patients with both high sMICA and sMICB levels also had markedly decreased survival rates. These findings suggest the utility of sMICB levels as a marker for tumour progression. Future studies should examine a greater number of OSCC patients with increased sMICB levels. Careful follow-up is recommended for patients with both high sMICB and sMICA levels, given the possibility for cancer recurrence.

Acknowledgements

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