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An association between the *MICA-A5.1* allele and an increased susceptibility to oral squamous cell carcinoma in Japanese patients

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BACKGROUND: Recently, a new polymorphic gene family called the major histocompatibility complex class I chain-related gene A (*MICA*) was discovered about 40 kb centromeric to HLA-B gene. The MICA protein, expressed on epithelial cells and many kinds of tumor cells, serves to regulate immune function. The MICA protein is thought to activate immune function on mucosal tissue by binding to NKG2D which is expressed on most natural killer cells, CD8 positive T cells, and gamma delta T cells. An association between *MICA* gene polymorpisms and the development of oral squamous cell carcinoma (OSCC) has also been reported.

OBJECTIVE: This study was designed to test this association in Japanese patients with OSCC.

METHODS: The $(GCT)_n$ polymorphisms of the *MICA* gene was investigated in 123 patients with OSCC and 188 normal controls using polymerase chain reaction amplification and denaturing polyacrylamide gel electrophoresis.

RESULTS: Five alleles, namely *A4*, *A5*, *A6*, *A9*, and *A5.1*, were found in both groups. The phenotype frequency of the *MICA-A5.1* allele was significantly higher in patients with OSCC when compared with normal controls (OR 1.707, 95% CI 0.76–3.45, $P = 0.042$). Also, the microsatellite frequency of the *MICA-A5.1* allele was significantly higher in patients with OSCC compared with normal controls (OR 1.664, 95% CI 0.82–3.42, $P = 0.021$). Lastly, the frequency of the *MICA-A5.1* allele was significantly higher in those with lymph node metastasis from OSCC compared with normal controls (OR 2.605, 95% CI 1.14–5.27, $P = 0.026$).

CONCLUSIONS: These results suggest that the *MICA-A5.1* allele may be associated with an increased susceptibility to OSCC in Japan.

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Introduction

The human leukocyte antigen (HLA) system plays an important role in the cellular immune response to viral and tumor antigens (1, 2). The HLA region, located on chromosome 6p21.3, encompasses a 4000-kb segment that has evolved through repeated gene duplication and conversion (3). Studies have shown that HLA is associated with tumor susceptibility, lymph node metastasis, and induction of cytotoxic T lymphocytes and natural killer cells in patients with head and neck carcinoma (4, 5).

The major histocompatibility complex (MHC) class I chain-related (*MIC*) gene family consists of five members: MICA, MICB, MICC, MICD, and MICE (6, 7). Of these, only MICA and MICB encode expressed transcripts. *MICA* gene is located about 40-kb centromeric to the *HLA-B* gene. Recently, it was shown that the polymorphic MICA protein is mainly expressed on epithelial cells, keratinocytes, freshly isolated monocytes, and many kinds of tumor cells (8, 9). It was also shown that the MICA protein is involved in the regulation of immunological function, especially under conditions of stress. The MICA protein is thought to activate immune function on mucosal tissue by binding to NKG2D, which is expressed on most natural killer cells, CD8 positive T cells, and gamma delta T cells (10, 11).

Recent studies have demonstrated a triplet repeat microsatellite polymorphism $(GCT)_n$ in the transmembrane (TM) region (exon five) of the *MICA* gene. This polymorphism consists of five alleles: four, five, six, or nine repetitions of GCT or five repetitions of GCT with one additional nucleotide insertion (G) designated as *A4*, *A5*, *A6*, *A9*, and *A5.1* respectively (12, 13). These polymorphic GCT repeats are associated with particular

alleles of the *HLA-B* and *MICA* genes and have been implicated in diseases such as ankylosing spondylitis (14), Bechet disease (3), rhumatoid arthritis (15), and many infectious diseases. Interestingly, MICA protein expression has been demonstrated in several carcinomas of epithelial cell origin as well as in rejected or injured renal and pancreatic allografts (16). However, no studies have yet found an association between the length of the *MICA* gene polymorphism and the presence of tumors such as skin cancer (17), cervical cancer (18), and cervical intraepithelial neoplasia (19).

Oral squamous cell carcinoma (OSCC) is a solid tumor originating from epithelial cells. It is the sixth most common cancer globally (20). The incidence of this disease varies dramatically worldwide; the relative frequency of OSCC amongst all malignancies ranges between less than 1% to over 40% (21). The annual incidence of OSCC in Japan is approximately 6000 and this number tends to vary depending on tobacco and alcohol consumption patterns (22).

While the correlation of *MICA* gene polymorphisms with OSCC has been suggested, further investigation of confounders such as genetic predisposition and clinico-pathological factors are needed (23–25). In this study, we attempted to demonstrate the association of various *MICA* gene polymorphisms with the development of OSCC by comparing these patients to unrelated normal controls.

Table 1 Clinical parameters of 123 patients with OSCC

Parameters	*Frequency (%)*
Mean age (years)	63.8 (range 32–93)
Gender (male/female)	58/65 (47/53)
Tumor size	
T1	15 (12.2)
T2	58 (47.2)
T3	36 (29.3)
T4	14 (11.3)
Lymph node status	
N0	47 (38,2)
N1	56 (45.5)
N2	17 (13.8)
N3	3 (2.5)
Disease stage	
I	15 (12.2)
II	23 (18.7)
III	63 (51.2)
IV	22 (17.9)
Tumor differentiation	
Well	61 (49.6)
Moderate	46 (37.4)
Poor	16 (13.0)
Tumor location	
Tongue	86 (69.9)
Upper gingival	16 (13.0)
Lower gingival	13 (10.6)
Buccal mucosa	8 (6.5)

Tumor size, lymph node status, and disease stage grouping were classified according to the 1997 International Union Against Cancer criteria.

Materials and methods

Subjects

The study subjects were all Japanese. One hundred twenty-three patients (58 males with a mean age 63.5 years and 65 females with a mean age 64.5 years) with OSCC and 188 unrelated normal controls (86 males with a mean age of 39.7 years and 102 females with a mean age 35.9 years) were enrolled in this study. These patients were seen at the Department of Oral and Maxillofacial Surgery in Nara University Hospital between 1999 and May 2006. Clinical information (including age, gender, tumor size, lymph node status, disease stage grouping, histological grade, tumor location, and clinical outcome) was obtained from the clinical records (Table 1). Primary tumor size, lymph node status, and disease stage grouping were classified according to the 1997 UICC criteria (26). One hundred eighty-eight normal controls were healthy volunteers and selected from among people who came to the clinic for routine physical checkups, non-neoplastic minor operations, or maxillofacial trauma. Those with autoimmune disorders, hematologic disorders, immunogenetic diseases, or previous malignancy were excluded.

Peripheral blood was collected after the details of this study were explained to each subject and consent to genetic screening was obtained. The Ethics Committee of the Nara Medical University approved this study (No. 40).

Oligonucleotides

High molecular weight genomic DNA was isolated from peripheral blood cells using a phenol–chloroform extraction of sodium dodecyl-lysed and proteinase-K-treated cells as described previously (27). To analyze the microsatellite repeat polymorphisms in the TM region of the *MICA* gene, we designed PCR primers flanking the TM region (MICA5F, 5′-CCTTTT TTTCAGGG AAA GTGC-3; MICA5R, 5′-CCTTAC CATCTCCA GAAACTGC-3′) (27). The MICA5R primer corresponds to the intron four and exon five boundary region. The MICA5F primer is located in intron five. MICA5R was labeled at the 5′-end with the fluorescent amidite reagent, 6-FAM amidite (Applied Biosystems, Foster City, CA, USA).

PCR amplification of the TM region of the MICA gene

A total of 100–200 ng of high molecular weight DNA were subject to PCR amplification of the TM region

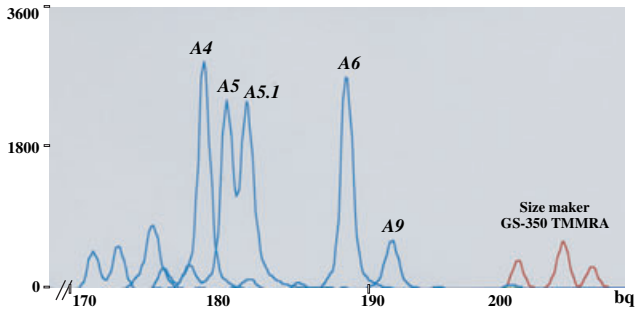

Figure 1 Digitalized electropherograms. Five distinct alleles *A4* (179bq), *A5* (182bq), *A5.1* (183bq), *A6* (185bq), *A9* (194bq) corresponding variable number of GCT repeats.

Table 2 Microsatellite allele frequencies of *MICA* genes in various nationalities

MICA TM-region	*Taiwanese (23)*	*Dutch (37)*	*Italian (13)*	*Japanese*	
				Prior studies (13)	*Present study*
A4	18 (11.0)	44 (8.9)	2 (9.6)	35 (17.0)	63 (16.8)
A5	64 (39.0)	56 (11.3)	6 (21.2)	65 (31.6)	112 (29.8)
A5.1	41 (25.0)	256 (51.8)	3 (11.5)	19 (9.2)	45 (12.0)
A6	8 (4.9)	69 (14.0)	10 (40.4)	53 (25.7)	98 (26.0)
A9	33 (20.1)	69 (14.0)	5 (17.3)	34 (16.5)	58 (15.4)
Total	164 (100)	494 (100)	26 (100)	206 (100)	376 (100)

Values in parenthesis are percentages. TM, transmembrane region.

in the *MICA* gene. PCR amplification was carried out in a PCR system. The reaction mixture was subjected to denaturation at 94°C for 2 min followed immediately by 32 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C.

Typing of triplet repeat polymorphism in the TM region of the MICA gene

To determine the number of the triplet repeats in the TM region of the *MICA* gene, the amplified products were denatured for 5 min at 100°C and then mixed with formamide containing a stop buffer. This product was subsequently electrophoresed on 6% polyacrylamide gels containing 8 M urea in an automated DNA sequencer. The number of microsatellite repeats was estimated using the Genescan software (Applied Biosystems, Foster City, CA, USA). This method employs the local Southern method with both a size standard marker of 350 TAMRA as well as with the PCR products of the B-cell lines that had been determined for the triplet repeat polymorphisms by nucleotide sequence determination as described before (3, 27) (Fig. 1).

Assignment of alleles

Allele designation was based on the number of repeat units present in the amplified allelic fragments of the TM region of the *MICA* gene. The alleles consisting of four, five, six, and nine repetitions of GCT and the allele consisting of five repetitions of GCT with one additional nucleotide insertion (G) were designated as *A4*, *A5*, *A6*, *A9*, and *A5.1* respectively (3). Their amplified sizes were 179 bp (*A4*), 182 bq (*A5*), 183 bq (*A5.1*), 185 bq (*A6*), and 194 bq (*A9*). There are five types of homozygosity and 10 types of heterozygosity. These diploid MICA genotypes were classified as *A4–A4*, *A4–A5*, *A4–A5.1*, *A4–A6*, *A4–A9*, *A5–A5*, *A5–A5.1*, *A5–A6*, *A5–A9*, *A5.1–A5.1*, *A5.1–A6*, *A5.1–A9*, *A6–A6*, *A6–A9*, and *A9–A9*.

Statistical analysis

Microsatellite allele frequencies, phenotype frequencies, and genotype frequencies were estimated by direct counting. The significance of the distribution of alleles between the patients with OSCC and normal controls was tested using the Chi-squared (χ^2) method with continuity correction and Fisher's exact probability test (*P*-value test). A statistically significant difference was defined as $P < 0.05$. The odds ratio (OR) was calculated using the cross product ratio. An OR with a 95% confidence intervals (CI) was calculated as an estimation of the relative risk of OSCC.

Results

All microsatellite allele frequencies, phenotype frequencies, and genotypes frequencies were observed in the 123 patients with OSCC and 188 normal controls. In the normal controls, the MICA microsatellite allele frequency was calculated: 16.8% were *A4*, 29.8% were *A5*, 12.0% were *A5.1*, 26.0% were *A6*, and 15.4% were *A9*. The frequencies were different in various geographical areas (Table 2). According to a prior study by Mizuki, in the Japanese population the *MICA-A5* allele was the most common, with a microsatellite allele frequency of 31.6% (3). The *MICA-A5.1* allele was observed in only 9.2% of the Japanese population. The microsatellite allele frequencies of normal controls in this study concurred with those of previous reports (Table 2).

There was no significant difference observed in the frequencies of *MICA-A4*, *A5*, *A6*, and *A9* alleles between the normal controls and the patients with OSCC. However, the phenotype frequency of the *MICA-A5.1* allele was significantly higher in patients with OSCC when compared with the normal controls (OR 1.707, 95% CI 0.76–3.45, $P = 0.042$). Furthermore, the microsatellite allele frequency of *MICA-A5.1* was significantly higher in patients with OSCC when compared with the normal controls (OR 1.664, 95% CI 0.82–3.42, $P = 0.021$) (Tables 3 and 4). There were 41 cases of homozygosity (33.3%) and 82 cases heterozygosity (66.7%) in patients with OSCC; there were 53 cases of homozygosity (28.2%) and 135 cases heterozygosity

Table 3 Phenotype frequencies of *MICA* genes in OSCC and in controls

MICA TM-region	*OSCC (n = 123)*	*Control (n = 188)*	*OR*	*95% CI*	*P-value*
A4	34 (27.6)	55 (29.3)			
A5	49 (39.8)	93 (49.5)			
A5.1	38 (30.9)	39 (20.7)	1.707	0.76–3.45	0.042*
A6	52 (42.3)	85 (45.2)			
A9	32 (26.0)	52 (27.7)			

Values in parenthesis are percentages. OR, odds ratio; CI, confidence intervals; TM, transmembrane region. Statistically significant difference at $*P < 0.05$.

Table 4 Microsatellite allele frequencies of *MICA* genes in OSCC and in controls

MICA TM-region	*OSCC*	*Control*	*OR*	*95% CI*	*P-value*
A4	39 (15.9)	63 (16.8)			
A5	59 (24.0)	112 (29.8)			
A5.1	49 (19.9)	45 (12.0)	1.664	0.82–3.42	0.021*
A6	64 (26.0)	98 (26.0)			
A9	35 (14.2)	58 (15.4)			
Total	246 (100)	376 (100)			

Values in parenthesis are percentages. OR, odds ratio; CI, confidence intervals; TM, transmembrane region. Statistically significant difference at $*P < 0.05$.

Table 5 Genotype frequencies of *MICA* genes in OSCC and in controls

Phenotype	*OSCC*	*Controls*	*OR*	*95% CI*	*P-value*
A4–A4	5 (4.1)	8 (4.3)			
–A5	14 (11.4)	14 (7.4)			
–A5.1	5 (4.1)	8 (4.3)			
–A6	5 (4.1)	14 (7.4)			
–A9	5 (4.1)	10 (5.3)			
A5–A5	10 (8.1)	20 (10.6)			
–A5.1	4 (3.3)	8 (4.3)			
–A6	15 (12.2)	31 (16.5)			
–A9	6 (4.9)	20 (10.6)			
A5.1–A5.1	11 (8.9)	6 (3.3)	2.979	1.73-6.38	0.029*
–A6	10 (8.1)	14 (7.4)			
–A9	8 (6.4)	3 (1.6)	4.290	2.31–8.52	0.048*
A6–A6	12 (9.8)	13 (6.9)			
–A9	10 (8.1)	13 (6.9)			
A9–A9	3 (2.4)	6 (3.2)			
Total	123 (100)	188 (100)			

Values in parenthesis are percentages. OR, odds ratio CI, confidence intervals. Statistically significant difference at $*P < 0.05$.

(71.8%) in normal controls. The MICA genotype frequencies of the *A5.1–A5.1* and *A5.1–A9* were significantly higher in patients with OSCC when compared with normal controls (*A5.1–A5.1*: OR 2.979, 95% CI 1.73–6.38, $P = 0.029$, *A5.1–A9*: OR 4.290, 95% CI 2.31–8.52, $P = 0.048$) (Table 5).

We then assessed whether the *MICA-A5.1* allele contributes to disease risk after the clinico-pathological factors such as age, gender, tumor location, tumor differentiation, and the development of lymph node metastasis were included in the analyses. Patients with OSCC that had the *MICA-A5.1* allele had significantly increased lymph node metastasis compared with patients with OSCC that did not have the *MICA-A5.1* allele (OR 2.605, 95% CI 1.14–5.27, $P = 0.026$) (Table 6).

Discussion

MIC genes are a newly reported gene family that map to the region of HLA class I genes and consist of five members: MICA, MICB, MICC, MICD, and MICE. Only MICA and MICB have an open reading frame and encode for MIC molecules (28). The *MICA* gene is located about 40-kb centromeric to the *HLA-B* gene (6, 7). Expression of the MICA protein is regulated by a promoter heat shock element similar to those of other heat shock protein genes (8). The MICA protein is often expressed on monocytes, endothelial cells, fibroblasts, and keratinocytes, especially under stress conditions (4, 9). It may play a role in the immune response. Furthermore, the MICA protein is also frequently expressed in many progressing tumors, including lung, gastric, renal, colon, ovarian, and oral carcinomas as well as melanomas (29). This suggests that the MICA molecule might be functionally impaired in carcinogenesis and thereby it may promote immune evasion (29).

MICA proteins function as ligands for the stimulatory C-type lectin-like NKG2D receptor, which is expressed by NK cells, alpha–beta CD8 positive T cells, and gamma–delta T cells (30).The NKG2D signaling has been shown to have an important role in NK- and T-cell-mediated innate responses to tumors. MICA proteins are thought to have the opposite effect because they evade immune surveillance through blocking the NKG2D receptor (31, 32). They do this via activation of heat shock transcription elements in the promoters of the corresponding genes in an event known to

Table 6 The *MICA-A5.1* allele and clinico-pathological characteristics of OSCC

Parameters	*MICA-A5.1, frequency (%) (n = 38)*	*No MICA-A5.1, frequency (%) (n = 85)*	*OR*	*95% CI*	*P-value*
Age, mean (range)	68.0 (range 41–92)	61.9 (range 32–93)			
Gender (male/female)	18/20	40/45			
Tumor location					
Tongue	30 (78.9)	56 (65.8)			
Upper gingiva	3 (7.9)	13 (15.3)			
Lower gingiva	3 (7.9)	10 (11.8)			
Buccal mucosa	2 (5.3)	6 (7.1)			
Tumor differentiation					
Well	20 (52.6)	44 (51.8)			
Moderate	11 (28.9)	36 (42.4)			
Poor	7 (18.4)	5 (5.8)			
Lymph node metastasis positive	29 (76.3)	47 (55.3)	2.605	1.14–5.27	0.026*

OR, odds ratio, CI, confidence intervals. Statistically significant difference at $*P < 0.05$.

accompany transformation (29). MICA proteins can thus promote tumor growth.

In this study, we identified five microsatellite alleles in the MICA TM region. The variable number of GCT repeats in exon five of the TM region encodes tandem repeats of the alanine residue (13). Four of them consist of four, five, six, and nine repetitions of GCT/AGC. Conversely, one allele (the *MICA-A5.1* allele) contains five triplet repeats plus one additional nucleotide insertion (GGCT/AGCC). This causes a frame shift mutation which results in premature termination by the stop codon (TAA) in the TM region (3). The TM region of this truncated MICA protein is not rich in hydrophobic amino acid residues and has a shorter TM region containing only two alanines and no cytoplasmic tail (3, 33). Thus it may be unable to fix on the cellular membrane. Instead, the protein translated from the *MICA-A5.1* allele is likely to be soluble and so is probably secreted.

It is known that there are other soluble HLA antigens in humans similar to the mouse H-2 class I molecule (3). For instance, the *MICA-A5.1* allele may have a similar function in humans as the H-2 class I molecule does in the mouse. In fact, the *MICA-A5.1* allele has been reported as a genetic maker of adult-onset type 1 diabetes mellitus (34), celiac disease (35), and psoriasis vulgaris (36).

The present study is the first to evaluate the relationship between the *MICA* gene and the development of OSCC in Japanese people. This study investigated the allelic distribution of microsatellite polymorphisms in the TM region of the *MICA* gene among patients diagnosed with OSCC and among normal controls. Our results demonstrate that Japanese carrying the *MICA-A5.1* allele have a higher risk of developing OSCC. They also show that the *MICA-A5.1* allele is associated with an increase in lymph node metastasis among patients with OSCC.

Internationally, the *MICA* gene frequency varies significantly. For example, Taiwanese patients with OSCC showed a significant increase in the frequency of the *MICA-A6* allele and Dutch patients with OSCC showed a significantly decreased frequency of the *MICA-A9* allele repeat (23, 37). Different frequencies of MICA repeats in different populations might explain various observed epidemiologic results in Taiwanese, Dutch, and Japanese OSCC patients.

Prior studies have demonstrated that the MICA protein is constitutively expressed within epithelial tumor cells and it is up-regulated in response to stress (38, 39). It is a ligand for the NKG2D receptor on both CD8 positive T cells and NK cells, reducing the threshold for lysis (10, 24). OSCC may up-regulate MICA protein expression in response to physical stressors such as anoxia; this response may make them susceptible to immune attack, resulting in better control of tumor growth and a more favorable prognosis (25, 31, 39).

In conclusion, this study of genetic markers in Japanese patients with OSCC demonstrates that the *MICA-A5.1* allele may be linked to an increased susceptibility to OSCC. The *MICA-A5.1* allele was also found to be associated with the development of lymph node metastasis. Despite the small sample size (peripheral blood), there was a large number of both normal control and patients with OSCC enrolled in this study. Thus, we were able to demonstrate clear associations between the *MICA* genes and susceptibility to OSCC.

To further this research, we will next monitor the response to therapy and the prognostic relevance of MICA in OSCC diseases by examining the amount of soluble MICA in the serum. Such results will help in the development of both genetic diagnostic modalities and gene therapy for OSCC in the future.

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