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Polymorphism of the MICA gene and risk for oral submucous fibrosis

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BACKGROUND: Oral submucous fibrosis (OSF), an insidious, pre-cancerous, chronic disease that may affect the entire oral cavity and sometimes extend to the pharynx has been reported to be associated with immune function. The major histocompatibility complex (MHC) class I chain-related gene A (MICA) is expressed by keratinocytes and other epithelial cells, and its encoded protein interacts with γ/δ T-cells localized in the submucosa. The MICA gene has a triplet repeat (GCT) polymorphism in the transmembrane domain resulting in five distinct allelic patterns.

METHODS: We analyzed MICA polymorphism in 80 OSF patients and 351 randomly selected unrelated controls by using the ABI Prism 377-18 DNA sequencer (Applied Biosystems) to analyze the sample DNA PCR products. The number of microsatellite repeats was estimated with Genescan 672 software (Applied Biosystems) with a standard size marker of GS-350 TAMRA.

RESULTS: The phenotype frequency of allele A6 of MICA in subjects with OSF was significantly higher than that in controls (OR = 3.48; 95% CI = 1.8–6.71; *P* = 0.0002), as was the frequency of the allele (OR = 2.65; 95% CI = 1.44–4.86; *P* = 0.001).

CONCLUSION: The results suggest that allele A6 in MICA might confer a risk for OSF.

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Introduction

Oral submucous fibrosis (OSF) is an insidious, pre-cancerous, chronic disease that may affect the entire oral cavity; it sometimes extends to the pharynx (1, 2). OSF is characterized by mucosal rigidity of varying intensity because of fibroelastic transformation of the juxta-epithelial layer. A subepithelial inflammatory reaction is followed by fibroelastic change of the lamina propria with epithelial atrophy (3). This leads to restricted mouth opening and inability to eat (2). The presence of palpable fibrous bands is a requisite diagnostic criterion for this condition, which may avoid misdiagnosing other mucosal conditions, such as those related to anemia, as OSF. This misdiagnosis has been prevalent in field studies.

OSF is predominately seen in people in south Asian countries (4), or south Asian immigrants to other parts of the world (5, 6). It is now a public health issue in many parts of the world, including the UK (7), South Africa (8), and many south-east Asian countries (6, 9, 10).

Although the available epidemiological evidence indicates that chewing of the areca nut is an important risk for developing OSF (9, 11–13), not all chewers develop the disease. Other information, however, indicates that the disease is not necessarily dose–response-related (14). Cessation of the habit also does not influence the characteristics of the disease once it is established (15). Genetic predisposition might explain such individual variability (7).

Several reports have suggested that molecular mimicry may explain the association between human leukocyte antigen (HLA) and autoimmune disease (16). Canniff et al. performed HLA tissue typing of OSF and observed that the frequencies of HLA A10, DR3, and DR7 were significantly different from a control group (7), with an increase in DR3 antigen in OSF and also the presence of serum immunoglobulins and autoantibodies. In addition, other antibody studies and HLA-typing on individuals with OSF have led to the conclusion that the origin of the disease is multifactorial and that it may be an autoimmune condition with a genetic predisposition (7, 8, 16).

In the classical class I peptide-presenting molecules, a new polymorphic gene family has been proposed – the major histocompatibility complex (MHC) class I chain-related genes (17). This family consists of five genes: MHC class I chain-related gene A (MICA), gene B (MICB), gene C (MICC), gene D (MICD), and gene E (MICE). MICC, MICD, and MICE are pseudogenes, while MICA and MICB encode mRNA under stress situations (18, 19).

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Table 1 Phenotype frequencies of MICA gene exon 5 in various races

Allele	*Taiwanese (n = 351; %)*[a]	*Japanese (n = 103; %)*[b]	*Spanish (n = 342; %)*[b]	*Swedish (n = 153; %)*[b]
A4	31	30	23	26
A5	50	52	21	56
A5.1	36	17	43	66
A6	8	46	55	9
A9	18	31	29	18

[a]Reference (29, 42).
[b]Reference (29).

MICA encodes molecules similar to MHC class I antigens and may share the capacity to bind peptides or other short ligands (20). Recently, it was shown that this polymorphic molecule is mainly expressed by epithelial cells (21) and interacts with γ/δ T-cells, which dominate lymphocyte populations isolated from the lamina propria (19). Expression of MICA by epithelium and its recognition by γ/δ T-cells suggest that it may play a role in immune surveillance and direct induction of mucosal immunity (22). Stress-inducible MICA is found to bind to NKG2D, a natural killer cell receptor. Nearly 50 MICA alleles have been described, although only 15 alleles have been officially assigned so far (23).

MICA has a triplet repeat microsatellite polymorphism $(GCT)_n$ in the transmembrane region in exon 5 of the gene (20). According to the putative open-reading frame of MICA cDNA, the microsatellite encodes polyalanine and therefore the number of alanine residues differs by the number of triplet repeats. For instance, an A6 allele is defined as containing six GCT repeats in this region. In addition, one specific allele (A5.1) containing five triplet repeats plus one additional nucleotide insertion (GGCT), causes a frame-shift mutation and results in premature termination by the generation of a novel stop codon (TAA) in the transmembrane region. This allele may encode a secreted, soluble MICA molecule. The alleles of MICA vary among individuals, and hence this microsatellite can be used as an informative polymorphic marker for genetic mapping and for disease susceptibility analysis. The frequencies of MICA alleles in exon 5 transmembrane regions in Caucasian, Japanese, and Spanish populations have been described (Table 1). It appears to vary greatly among different populations (24). However, the global frequency and racial discrepancies have not been fully elucidated. Investigation of a possible correlation of MICA polymorphism with a genetic predisposition to OSF might clarify the molecular events involved in the pathogenesis or the immuno-defense against OSF. In this study, we compared the allelic frequency of MICA in OSF patients and normal individuals.

Materials and methods

Subjects

Eighty consecutive males, pathologically proved as OSF patients recruited from the Oral and Maxillofacial Department at Taipei Mackay Memorial Hospital, were enrolled between January 1999 and December 2001. None of the patients had oral cancer. Three hundred and fifty-one control subjects were selected from people who came for routine physical check-ups, non-neoplastic minor operations, or maxillofacial trauma. Those with autoimmune disorders, blood diseases, and previous malignancy were excluded. After informed consent was obtained, blood was drawn from the subjects to extract genomic DNA. The Human Research Review Committee of Mackay Memorial Hospital approved this study.

Polymorphism analysis

A PCR-based polymorphism assay was used in this study.

DNA extraction

Genomic DNA was extracted from fresh or frozen peripheral blood leukocytes by standard techniques (23–25).

Primers

Primers flanking the transmembrane region were designed according to the reported sequence (17, 20). They were MICA5F: 5′-CCTTTTTTTCAGGGAAAGTGC-3′ and MICA5R: 5′-CCTTACCATCTCCAGAAACTGC-3′. The MICA5F primer corresponds to the intron 4 and exon 5 boundary regions, and MICA5R is located in intron 5 (20). MICA5R was 5′-end-labeled with fluorescent dye (Applied Biosystems, Foster City, CA, USA; 21, 24–26).

PCR

The amplification reaction mixture (15 μl) contained 50 ng genomic DNA, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.5 μM of each primer, and 0.5 unit ProzymeDNA polymerase (Protech Enterprise, Taipei, Taiwan). The PCR reaction was carried out in a GeneAmp PCR system (Perkin-Elmer Corporation, Foster City, CA, USA). The reaction mixture was subjected to denaturation at 95°C for 5 min followed by 10 cycles at 94°C for 15 s, 55°C for 15 s, 72°C for 30 s, then by an additional 20 cycles at 89°C for 15 s, 55°C for 15 s, 72°C for 30 s, and by a final extension at 72°C for 10 min.

Gel electrophoresis

The PCR products were denatured for 5 min at 100°C, mixed with formamide-containing stop buffer, and subjected to electrophoresis on 4% polyacrylamide gel containing 8 M urea in an ABI Prism 377–18 DNA sequencer (Applied Biosystems). The number of microsatellite repeats was estimated automatically with Genescan 672 software (Applied Biosystems) with a standard size marker of GS-350 TAMRA (*N*,*N*,*N*,*N*-trtramethy-l-6-carbohydroxyl rhodamine; Applied Biosystems; 26). Alleles were designated according to the classification of Mizuki et al. (21). Their amplified sizes are 179 bp (A4), 182 bp (A5), 183 bp (A5.1), 185 bp (A6), and 194 bp (A9). At least two independent experiments were performed on each sample to assure that the analysis was reproducible.

Statistical analysis

Evaluation of the Hardy–Weinberg equilibrium was performed by comparing observed and expected heterozygotes and homozygotes, as well as observed and expected genotypes, using Chi-square test analysis. Because the various

alleles are expressed dominantly, heterozygotes were counted as having the allelic phenotype. Phenotype or gene frequencies of OSF and controls were compared by Chi-square test analysis with Yates' correction where appropriate (one expected number <5). OSF and controls positive for a factor were compared by the same test using a Prism 4 statistical program (GraphPad Software, Inc.). Corrected *P*-values (pc) were calculated using the Bonferonni inequality method for the number of comparisons. Statistical significance was defined as $pc < 0.05$. Only data with a $P < 0.05$ are shown.

Results

Characteristics of subjects

The age range of 80 patients with OSF was 21–67 years (mean ± SD = 39.47 ± 7.7). The controls ages ranged from 22 to 71 years (mean ± SD = 42.1 ± 10.7). A marked gender difference (male:female = 79 : 1) was noted, which is consistent with the distribution of OSF in the Taiwanese population (6). The gender distribution of the controls was 185 : 166 (male:female).

MICA gene polymorphism

The distribution of MICA genotypes in both groups was in Hardy–Weinberg equilibrium, i.e. the observed and expected figures did not differ. Figure 1(A) shows representative electrophoretograms of the PCR products from a pre-test examination. The sequence of each type of homogenous allele was confirmed by direct sequencing (detailed analysis not shown). Figure 1(B) is a representative diagram illustrating a control (#135) and OSF sample (OSF28). The phenotype frequency of allele A6 in patients with OSF was significantly higher than in controls (OR = 3.48; 95% CI = 1.8–6.71; $P = 0.0002$; Table 2), as was the allele frequency of allele A6 (OR = 2.65; 95% CI = 1.44–4.86; $P = 0.001$; Table 3). No significant differences in the frequency of other MICA alleles in OSF were observed. Among the controls, no significant differences in allelic types were observed in individuals aged ≥42 years vs. <42 years or in males vs. females.

Discussion

Our study investigated the allelic distribution of microsatellite polymorphism in the transmembrane region of the MICA gene among OSF patient in Taiwan. In this study, we provide novel evidence indicating a higher frequency of the MICA A6 allele in OSF patients in comparison with normal individuals. MHC class I molecules are important in the efferent limb of immunity, which is thought to function in destroying cells bearing foreign antigens. Cytotoxic T-cells

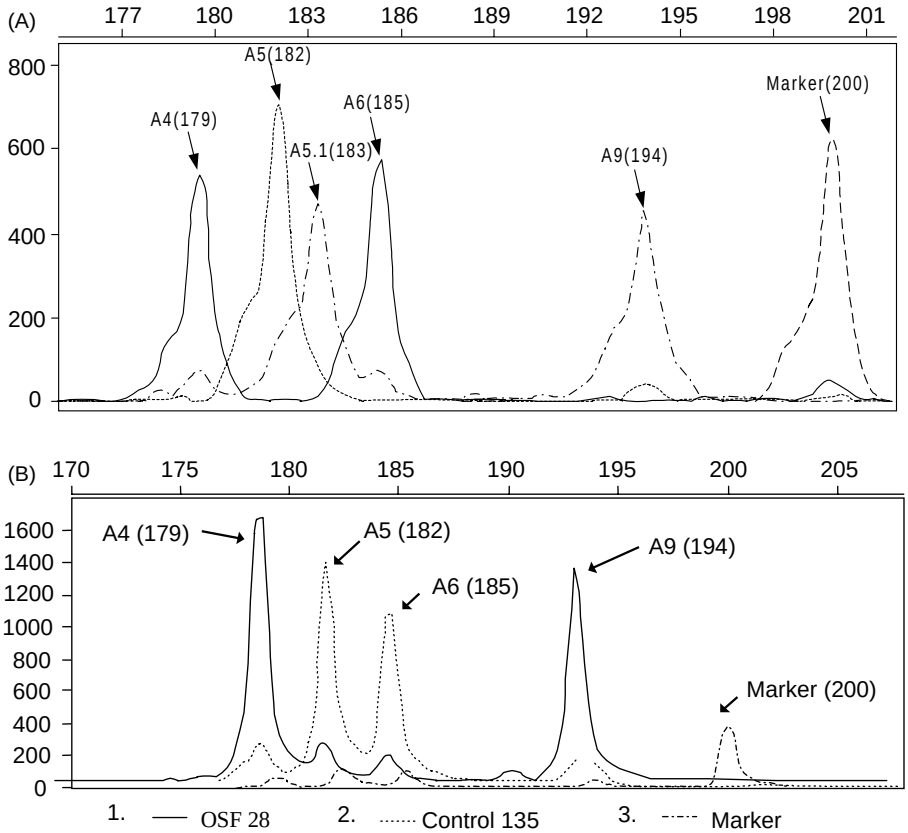

Figure 1 Digitalized electropherograms. (A) Overlapping representative diagrams exhibiting the migration of the PCR products of each allele in a pre-test running. *X*-scale, molecular size; *Y*-scale, intensity of fluorescence; number in parentheses, size of PCR product. Note the distinct separation of each peak. (B) Comparison of the migration of two examined samples. Liu 128, an OSF subject displaying A4 and A9 alleles; control 135, a control subject displaying A5 and A6 alleles. The minor bands in some peaks are the artifacts of overlapping images, which is automatically eliminated by the software in the final analysis.

Table 2 Phenotype frequencies of MICA gene exon 5 in OSF patients and controls

	OSF		*Control*						
Allele	*n = 80*	*%*	*n = 351*	*%*	*OR*	*95% CI*	χ^2	*P*	*pc*
A4	18	23	107	31	–	–	ns	–	–
A5	44	55	174	50	–	–	ns	–	–
A5.1	36	45	127	36	–	–	ns	–	–
A6	18	23	27	8	3.48	1.8–6.71	15.27	0.0002	0.001
A9	22	28	62	18	–	–	ns	–	–

OR, Odds ratio; CI, confidence interval; ns, not significant; pc, corrected *P*-value.

Table 3 Allele frequencies of the MICA gene exon 5 in OSF patients and controls

	OSF		*Control*						
Allele	*n = 160*	*%*	*n = 702*	*%*	*OR*	*95% CI*	χ^2	*P*	*pc*
A4	20	13	161	23	–	–	ns	–	–
A5	58	35	262	37	–	–	ns	–	–
A5.1	38	24	177	25	–	–	ns	–	–
A6	18	12	32	5	2.65	1.44–4.86	10.67	0.001	0.005
A9	26	16	70	10	–	–	ns	–	–

OR, Odds ratio; CI, confidence interval; ns, not significant; pc, corrected *P*-value.

recognize the MHC gene product and destroy the cell bearing it. The local immune response in tissues with a low transcription level of HLA class I is therefore influenced by HLA class II and presumably influenced by MICA gene products, mainly produced by epithelial cells (21).The MICA gene is located near HLA-B on chromosome 6 and is by far the most divergent mammalian MHC class I gene known. The MICA gene has recently been found to be significantly associated with susceptibility to certain diseases linked with the HLA-B locus (19, 20, 26–28). This suggests that susceptibility associated with the HLA-B locus might be a result of variation in MICA genotypes. Further study is needed to investigate the association between the MICA gene and HLA-B. It would be intriguing to conduct genotyping of MICA in order to assess its role in the pathogenesis of OSF. The frequency of the A6 allele was remarkably higher in the patients than in the controls. It does not seem plausible, however, that six alanines in a transmembrane domain could be solely responsible for such a difference in susceptibility. In addition, phenotypic incidence of A6 is nearly 50% in Japanese and Spanish (29; Table 1). It is possible that the high density of the A6 microsatellite allele in OSF populations may reflect linkage disequilibrium of the chromosomal locus with a certain allelic form of the MICA in the peptide-binding region. This might also interfere with the binding interaction with NKG2D, resulting in extensive flexibility.

Another intriguing question is the relation between OSF and natural killer cells. The normal patterns of target binding cells were seen in OSF, but there was a reduced number of active killer cells (30). NKG2D, a C-type lectin encoded by a member of the NK receptor gene complex, was reported to be the MICA receptor (31). In humans, NKG2D is expressed on most natural killer cells, γ/δ T-cells, and CD 8αβ T-cells (31). Ligands of NKG2D include the MHC class I homologues MICA and MICB, which function as signals of cellular stress (19, 27). These molecules are absent from most cells and tissues but can be induced by viral and bacterial infections and are frequently expressed in epithelial tumors (19, 32–34). MIC engagement of NKG2D triggers natural killer cells and co-stimulates antigen-specific effector T-cells (31, 33). Groh (35) recently showed that binding of MIC induces endocytosis and degradation of NKG2D. Systemic deficiency is associated with circulating tumor-derived soluble MICA, causing down-regulation of NKG2D and, in turn, severe impairment of tumor–antigen-specific effector T-cell responsiveness. This mode of T-cell silencing may promote tumor evasion of immunity and, by inference, compromise host resistance to infection (35).

γ/δ T-cells are a subset of T-cells expressing the T-cell receptor (TCR) γ/δ heterodimer (36) Although they constitute only about 5% of circulating T-cells, they are much more common in epithelial tissues, i.e. the skin, intestine, oral mucosa, lung, and female reproductive tract (37, 38). Profound alterations of peripheral blood T-lymphocyte subsets have been found in OSF, with an imbalance in the ratio of cells bearing the gamma and mu receptors functionally recognized as cells mediating suppressor and helper functions, respectively (39). The stress-induced expression of MICA and MICB and their recognition by diverse $V_\delta 1$ γδ T-cells may serve as an immune surveillance mechanism for the detection of damaged, infected, or transformed oral epithelial cells, or may stimulate T-cell secretion of growth factors for the maintenance of epithelial homeostasis (36). It is very likely that MICA plays a role in the pathogenesis of OSF via γ/δ T- and NK-cells. Experiments are being carried out in our laboratory to test this possibility.

The possible pre-cancerous nature of OSF was first described by Paymaster (40), who observed the onset of slowly growing squamous cell carcinomas in one-third of

such patients. These observations were subsequently confirmed by Pindborg (41). In our previous study of OSCC, we found an increased frequency of the MICA gene A6 allele (42). In the present study, the increased microsatellite polymorphism of MICA gene A6 in OSF patients was similar to that seen previously in OSCC patients (42). This further supports the pre-cancerous nature of OSF.

The novelty of the present study was in demonstrating the association between MICA allele A6 and OSF. The risk for OSF may be further clarified by genotyping of the HLA-B locus, as well as MICA gene fragments, in addition to the transmembrane domain, and understanding the MICA protein function as influenced by genetic polymorphism. Furthermore, sorting out the complicated interactions among MICA, NKG2D-NK cell, and γ/δ T-cells is also of importance in elucidating the pathogenetic mechanisms of OSF.

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