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The functional (−1171 5A → 6A) polymorphisms of *matrix metalloproteinase 3* gene as a risk factor for oral submucous fibrosis among male areca users

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BACKGROUND: Insertion/deletion (−1171 5A → 6A) polymorphisms in the promoter region of *matrix metalloproteinase 3* (*MMP3*) gene result in different transcriptional activities. MMP3 is able to degrade collagens types II, V, IX, and X, and other extracellular matrix. The functional promoter polymorphism of *MMP3* has been related to the susceptibility in some inflammatory diseases and metastasis of cancers.

METHODS: Oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC) are prevalent among Asian areca users. In this study, genomic DNA obtained from the blood of OSCC ($n = 150$), OSF ($n = 71$), and control non-diseased areca user ($n = 98$) in male were subjected to polymerase chain reaction (PCR)-based genotyping of *MMP3*.

RESULTS: The 5A genotype in *MMP3* promoter was observed more frequently in OSF group than in control group ($P = 0.01$). No significant difference was noted between OSCC and control groups on the 5A genotype frequency ($P = 0.18$). No association was found between 5A genotype in *MMP3* promoter and site or lymph node metastasis and stage of OSCC.

CONCLUSION: The results indicated that the 5A genotype of *MMP3* promoter was associated with the risk of OSF but not OSCC.

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Keywords: genotype; *matrix metalloproteinase 3*; mouth; oral submucous fibrosis

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Introduction

Breakdown control of extracellular matrix (ECM) is essential in many physiologic and pathologic conditions, such as embryonic development, morphogenesis, reproduction, tissue resorption, and remodeling (1, 2). Among enzymes that are capable of degrading basement membrane and ECM, matrix metalloproteinase (MMPs) might be the most important one associated with disease genesis. MMPs can be divided into five subgroups according to their structure and substrate specificity: collagenases, stromelysins, gelatinases, membrane-type MMPs, and other MMPs. So far, 23 MMPs have been identified in the MMP superfamily (3).

Tumors often induce fibroproliferative response to the adjacent stroma, which is thought to be a host defense to the wall of a tumor. Hence, degradation of ECM is crucial in tumor progression, such as metastasis and angiogenesis. A number of studies have demonstrated the correlation between MMP expressions and the progression of malignancies, including colorectal, basal cell, lung, head and neck, esophagus, breast, bladder, and liver cancers (4–14). MMP3 (stromelysin-I) is expressed by fibroblast as well as epithelial cells in culture and *in vivo* (15). MMP3 degrades types II, V, IX, and X collagens, proteoglycans, gelatin, fibronectin, laminin, and elastin (16, 17). MMP3 can activate other MMPs, including collagenase, matrilysin, and gelatinase B. MMP3 is able to lyse basal membrane and induce other MMPs that are involved in invasion and metastasis (15, 18). Studies on MMP3 expressions in early stage of oral squamous cell carcinoma (OSCC) also found a positive correlation among tumor size, depth of tumor invasion, diffuse invasive mode, and incidence of lymph node metastasis (LNM; 19).

Expression of *MMP3* is regulated at the transcription level where the promoter region of the gene responds to growth factors, cytokines, and some environmental factors associated with ECM. A single adenine insertion/deletion polymorphism (5A/6A) at position −1171

of the *MMP3* promoter region causes different transcription of *MMP3*. *In vitro* assays of promoter activity showed that the 5A allele had a two-fold higher promoter activity than the 6A allele (20). A study has revealed that such polymorphism linked to the higher risk of metastasis in breast cancer patients (21). Studies also demonstrated that the expression of MMP3 was correlated with the LNM in SCC of esophagus (4). Hashimoto et al. have proposed adverse evidences showing that *MMP3* polymorphisms were not associated with the risk of head and neck SCC (HNSCC) (22).

In Taiwan, OSCC is prevalent due to the popularity of areca chewing. A report showed that more than 2 million areca chewers lived on this island (23). Thus, the identification of risk population using a polymorphic genetic marker is important. Oral submucous fibrosis (OSF) is a collagen-related disease exclusively linked to areca use. The incidence of OSF among areca users was reported as high as 11% in some population (24, 25). With a mean of 10-year follow-up period, the chance of having malignant transformation in OSF cases was reported to be 1–7.6% (26, 27). The imbalance between collagen deposition and degradation is a characteristic of OSF formation. *In vitro* studies revealed that arecoline, a major areca alkaloids, could affect oral mucosa fibroblast on collagen synthesis (28, 29). Arecoline and areca extract also retard the *in vitro* wound healing process of fibroblast that becomes vulnerable to tissue fibrosis (29). It was interesting to note that some patients developed OSF after a short areca-exposing time (30). This study was aimed to clarify whether the functional nucleotide polymorphism with different promoter activity of *MMP3* related to the susceptibility and the disease progression of OSCC and OSF.

Materials and methods

Subjects

A total of 150 OSCC patients and 71 OSF patients were enrolled in this study. Ninety-eight healthy areca chewers were selected for controls. All subjects were male areca chewers. Those with autoimmune disorders, blood diseases, and previous malignancies were excluded from the control group. The age of subjects are described in Table 1. The OSCC cases were further grouped by the event site (buccal mucosa vs. non-buccal mucosa), LNM (0 vs. >0) and clinical stages (I–III vs. IV). This study has been approved by an ethics reviewing board. Blood samples were drawn from the subjects after informed consents had been obtained. DNA was isolated by Qiagen Blood Mini Kit (Qiagen, Valencia, CA, USA) from leukocyte cell pellets, which were obtained from the buffy coat of the whole blood.

MMP3 genotyping

The *MMP3* promoter −1171 5A/6A polymorphism was determined by polymerase chain reaction (PCR)-based genotyping. The primers used to generate amplicons of 187-bp (for 5A), 188-bp (for 6A) or the mixture (for 5A/6A heterozygosity) were *MMP3* – sense: 5′-CCTGCCTCAACCTCTCAAAG-3′ and *MMP3* – antisense: 5′-CACTCTGTTCTCCTTGTCCTC-3′. The 5′-site of the sense primer was labeled with FAM fluorescence dye. The amplification reaction mixture (7.5 μl) contained 20 ng genomic DNA, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.5 unit Prozyme DNA polymerase (Protech Enterprise, Taipei, Taiwan) and 1X PCR buffer. The PCR was performed in three steps: first, 5 min at 94°C; then, 35 cycles of 30 s at 94°C, 30 s at 57°C, and 90 s at 72°C; lastly, 1 h at 72°C. The amplicons were denatured for 5 min at 100°C, and mixed with formamide-containing stop buffer, and then subjected to electrophoresis on 4% polyacrylamide gel containing 8-M urea in an ABI Prism 377-18 DNA sequencer (Applied Biosystem, Foster City, CA, USA). The fluorescence was detected automatically by GENESCAN 672 software (Applied Biosystem). At least two independent experiments were performed on each sample to assure the reliability of the analyses.

Table 1 Clinical parameters of study subjects

	Control	*OSCC*	*OSF*
n	98	150	70
Age (mean ± SD)	48.0 ± 9.0	51.2 ± 9.7	38.8 ± 10.6
Site			
Non-buccal mucosa	/	60	/
Buccal mucosa	/	90	/
LNM			
0	/	91	/
>0	/	59	/
Stage			
I–III	/	73	/
IV	/	77	/

OSCC, oral squamous cell carcinoma; OSF, oral submucous fibrosis; LNM, lymph node metastasis.

DNA sequencing

Direct sequencing of gel-purified PCR products (Qiaex II Gel Extraction Kit; Qiagen) was performed by a 377-18 DNA sequencer (Applied Biosystem) as instructed by the manufacturer.

Statistical analysis

The variants were analyzed with Fisher's exact test. Differences between the values were considered significant when $P<0.05$. The associations between the *MMP3* genotype and the risk of disease genesis were estimated by odds ratio (OR), and 95% confidence interval (CI) were calculated by unconditional logistic regression models using SPSS 12.0 (Chicago, IL, USA).

Results

The ages were 48.0 ± 9.0 in control subjects, 51.2 ± 9.7 in OSCC subjects and 38.8 ± 10.6 in OSF subjects (Table 1). The genotyping of *MMP3* promoter was performed by GENESCAN system. It distinguished homozygous 5A genotype (5A/5A), heterozygous genotype (5A/6A), and homozygous 6A genotype (6A/6A) patterns by the differential mobility of amplicons with different sizes. In some selected samples, homozygous

Table 2 Genotypes in *MMP3* promoter related to oral diseases

	n	*5A genotype frequency*	*5A/5A allelotype*	*5A/6A allelotype*	*6A/6A allelotype*	*P-value**	*OR*	*95% CI*	*Adjusted for age*		
									P-value	*OR*	*95% CI*
Control	98	0.07	1 (1)	12 (12)	85 (87)		1			1	
OSCC	150	0.10	0 (0)	31 (21)	119 (79)	0.17	1.70	0.84–3.45	0.18	1.63	0.78–3.26
OSF	70	0.15	1 (2)	19 (27)	50 (71)	0.02	2.62	1.20–5.71	0.01	3.21	1.33–7.89

*5A/5A and 5A/6A allelotypes vs. 6A/6A allelotype.
OR, odds ratio; CI, confidence interval; MMP, matrix metalloproteinase; OSCC, oral squamous cell carcinoma; OSF, oral submucous fibrosis.

Table 3 Genotypes in *MMP3* promoter related to OSCC

	n	*5A genotype frequency*	*5A/5A allelotype*	*5A/6A allelotype*	*6A/6A allelotype*	*P-value**
Non-buccal mucosa	60	0.11	0 (0)	13 (22)	47 (78)	0.84
Buccal mucosa	90	0.10	0 (0)	18 (20)	72 (80)	
LNM						
0	91	0.11	0 (0)	20 (22)	71 (78)	0.84
>0	59	0.10	0 (0)	12 (20)	47 (80)	
Stage						
I–III	73	0.10	0 (0)	15 (21)	58 (79)	1.00
IV	77	0.10	0 (0)	16 (21)	61 (79)	

*5A/5A and 5A/6A vs. 6A/6A.
MMP, matrix metalloproteinase; OSCC, oral squamous cell carcinoma; LNM, lymph node metastasis.

5A or homozygous 6A genotypes were confirmed by direct sequencing of the amplicons.

Frequencies of 5A genotype were 0.07 in controls, 0.10 in OSCC cases, and 0.15 in OSF cases (Table 2). No statistical significant difference on 5A genotype frequency was noted between control and OSCC subjects. A significant difference was found on 5A genotype frequency between control and OSF subjects with an age-adjusted OR (3.21; Table 2).

The OSCC cases were further subdivided according to their event site, neck LNM, and clinical stage. However, no significant difference found between OSCC with different sites, LNM status, and clinical stages (Table 3).

Discussion

The MMP activities are important for genesis or progression of malignancies. In previous studies, functional promoter polymorphisms in *MMP1* and *MMP2* genes, which might associated with the increase in expression, were found with the increased risk on developing OSCC but not OSF (31, 32). *MMP3* transcripts were more frequently observed in HNSCC than their matching normal tissues (5). In addition, OSCC with increased MMP3 expressions were significantly related to tumor invasion and LNM (33). The *MMP3* promoter −1171 5A genotype is associated with a higher *MMP3* transcription; however, we were unable to differentiate the incidence of 5A allele between controls and OSCC subjects. It seemed unlikely that functional genotype of *MMP3* promoter was related to the risk of areca-associated OSCC. In addition, *MMP3* polymorphism was not associated with the progression OSCC as reflected by LNM and advances in stage. Our data agreed with the findings that no significant difference observed in the *MMP3* genotypic status between HNSCC and control groups (22).

Fibrosis is a dynamic change between fibrogenesis and fibrolysis with a net output toward fibrogenesis. OSF is a disease, which can be induced by areca chewing producing disruption in stromal tissue and cytokine regulation. Similar to OSF, liver cirrhosis, radiation-induced fibrosis, and systemic sclerosis also show imbalance in fibrotic homeostasis. Under different areca exposure history, polymorphisms of genes associated with collagen homeostasis were reported to be correlated with the risks of OSF (34). In addition, polymorphisms of transforming growth factor (*TGF*)-α, MICA and CTLA-4 related to innate immune response were also found to associated with the risk of OSF (35–37). However, functional *MMP1* and *MMP2* genotypes did not appear to be risk factors for OSF. Murawaki et al. (38) identified the absence of association between MMP3 serum level and liver cirrhosis in a subset of cirrhotic child patients. It is interesting that autoantibody against MMP3 can be detected in patients with systemic sclerosis (39). In this study, a significant difference in *MMP3* genotypic polymorphism was found between control and OSF subjects in male areca chewers. Male areca chewers carrying 5A allele at −1171 in *MMP3* might have a greater than threefold risk for OSF in relation to subjects carrying the other genotype. It is interesting to discover that the functional genotypes in *MMP1–MMP3* confer differential risks of OSCC and OSF to areca chewers, suggesting that variations are involved with different combinations of MMPs on the pathogenesis.

The activities of MMPs should be balanced with tissue inhibitors of metalloproteinases (TIMPs) and could be affected by cytokines. In radiation enteritis, the

expressions of collagens, MMPs, and TIMPs, were simultaneously increased with a net effect of collagen deposition that led to transmural fibrosis of ileum (40). TGF-β1 could potentially induce *TIMP-1* expression in normal intestinal myofibroblasts and thus inhibit MMPs activities (41). It was also reported that cultured buccal mucosal fibroblast stimulated by arecoline and safrole increased mRNA expression of *TIMP-1* (42). Moreover, increase in *TIMP-1* expression was found in fibroblasts cultivated from OSF buccal mucosa (43). Although the results in this study implicated that *MMP3* transcription activity might link to the genesis of OSF in areca chewers, the evidences supporting the relationship between OSF formation and *MMP*s expression are uncertain. Because the increased expression of *TIMP-1* and its inhibitory activity toward MMP activity may underlie the pathogenesis of OSF, it is important to know whether the OSF subjects carrying 5A alleles in *MMP3* had the genotypic repression of *TIMP-1*.

This study indicated that *MMP3* functional promoter polymorphism is associated with the risk of OSF. The genotypic linkage among the collagen-related genes, *MMPs*, *TIMPs*, and cytokines related to OSF formation requires more information to specify the risk of OSF in areca chewers.

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