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Functional −1562 C-to-T polymorphism in *matrix metalloproteinase-9* (*MMP-9*) promoter is associated with the risk for oral squamous cell carcinoma in younger male areca users

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BACKGROUND: Circulating matrix metalloproteinase-9 (MMP-9) is a prognostic factor for gastric cancer and vascular diseases, and has been associated with head and neck cancers. The −1562 C-to-T polymorphism in *MMP-9* promoter (abbreviated *MMP-9* −1562 C>T polymorphism) leads to differential transcription, and is associated with increased susceptibility to neoplastic and vascular diseases. Thus, our aim was to determine whether a functional *MMP-9* polymorphism might also influence the risk or affect the progression of areca-associated oral cancers.

METHODS: Genomic DNAs were obtained from peripheral blood cells of male subjects with areca-associated oral squamous cell carcinoma (OSCC) (*n* = 192), oral submucosal fibrosis (OSF) (*n* = 73), and non-diseased areca users (*n* = 191). The PCR-based restriction fragment length polymorphism analysis was performed for *MMP-9* genotyping.

RESULTS: *MMP-9* −1562 C>T polymorphism was not associated with the risk of OSCC or OSF. However, when subjects were stratified by the median age, an association with the risk of OSCC was found in younger patients (*P* = 0.029). The T allele frequency was significantly higher in the subset of older patients with buccal mucosa OSCC than older patients with OSCC in counterpart locations. The joint *MMP-9* −1562 C>T and *MMP-3* −1171 5A>6A functional polymorphisms were not associated with OSCC risk or patient survival.

CONCLUSION: Aberrant *MMP-9* expression is closely related to tumor invasiveness and the prognosis of head and neck cancers. However, functional *MMP-9* −1562 C>T polymorphism is associated with OSCC risk only in younger areca chewers. The impact of aging or areca-related effect on this functional polymorphism should be elucidated.

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Introduction

Matrix metalloproteinase-9 (MMP-9) is one of a set of zinc-dependent endopeptidases capable of degrading components of the extracellular matrix (ECM). MMP-9 has three repetitive type II fibronectin domains, which allow it to bind to ECM components, such as gelatin, collagen, and laminin (1). Increased expression of this enzyme is seen in some neoplastic, cardiovascular and respiratory diseases (2–6). Recent studies revealed that the plasma level of MMP-9 is of both diagnostic and prognostic significance in coronary artery diseases (3, 7, 8) and renal diseases (9).

A functional cytosine (C) to thymidine (T) single nucleotide polymorphism at position −1562 in the *MMP-9* promoter was reported. Transient transfection and DNA-protein interaction assays indicated that T allele-associated promoter activity (due to the preferential binding of a putative transcriptional repressor protein) was higher than the C allele-associated promoter activity (10). Many studies further showed that this functional polymorphism was correlated to increased susceptibility to certain diseases. Individuals carrying the T allele were more susceptible to chronic

obstructive pulmonary disease (11), atherosclerosis (12), myocardial ischemia (13), and abdominal aortic aneurysm (14). In hepatitis C patients, the C allele's frequency was higher in the cirrhosis group than in the chronic hepatitis group (15, 16). The T allele-associated increase in basic MMP-9 might also provide weak protection against dementia by degrading the hallmark components of Alzheimer's disease (17). MMP-9 degrades type IV collagen, a major component of the basement membrane (18). The T allele in the *MMP-9* promoter was found to associate with the invasive phenotype or susceptibility to gastric cancers (19). Conversely, breast cancer patients carrying the T allele had better prognosis compared to homozygous C/C carriers (20). MMP-9 overexpression was noted in head and neck cancers (21).

Oral cancer is the fourth most common cause of male cancer mortality in Taiwan (22) and around 3300 new cases are diagnosed each year (23). The severity of this disease is most likely due to the high prevalence of the areca use. It is estimated that more than two million use areca in Taiwan and the number could be as high as 200 million worldwide (24, 25). The areca in Taiwan consists of the unripe areca nut, slaked lime, and betel inflorescence or leaf (24). Because of its cytotoxic, genotoxic, and mutagenic effects, arecoline (the main areca alkaloid) is considered to be the putative carcinogen (8, 26–28). Areca users are 28 times more susceptible to oral cancer than non-users (25). Previous studies demonstrated the association between promoter polymorphisms of *MMP-1* and *MMP-2* and the risks of oral squamous cell carcinomas (OSCCs) (29, 30). Since multiple MMPs can be involved in oral carcinogenesis, we studied whether the functional *MMP-9* −1562 C>T polymorphism might also influence the risk or affect the progression of areca-associated OSCCs.

Materials and methods

Subjects

In all, 192 OSCC patients, 73 submucosal fibrosis (OSF) patients, and 191 healthy areca chewers were enrolled in this study. All subjects were male areca chewers. The study protocol received Institutional Review Board approval from the Veterans General Hospital, Taipei. Blood samples were drawn from subjects after obtaining their informed consents. DNA was isolated from leukocyte pellets using the Qiagen Blood Mini Kit (Qiagen, Valencia, CA, USA). The clinical parameters of the subjects are described in Table 1. The OSCC cases were further stratified by lesion site: buccal mucosa (BM) *vs.* non-buccal mucosa (non-BM), nodal metastasis: $n = 0$ *vs.* $n > 0$, invasiveness: T1–3 vs. T4 and clinical stage: I–III vs. IV in subsequent analysis. The median ages of OSCC, OSF, and the control subjects (in years) were 51, 40, and 48, respectively.

MMP-9 genotyping

A 435-bp region spanning nucleotides −1809 to −1374 in the *MMP-9* promoter was amplified using forward primer 5′-GCCTGGCACATAGTAGGCCC-3′ and reverse primer 5′-CTTCCTAGCCAGCCGGCATC-3′ (12). The amplification reaction mixture (7.5 μl) contained 20 ng of genomic DNA, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.5 unit of Prozyme DNA polymerase (Protech Enterprise, Taipei, Taiwan), and 1x PCR buffer. The PCR reaction was performed for 5 min at 95°C and then for 35 cycles of 30 s at 95°C, 30 s at 61°C, and 90 s at 72°C with a final extension at 72°C for 10 min. Aliquots of the PCR product were mixed with 10 μl of reaction solution containing 1 μl of 10x enzyme buffer, 6 μl of 3dH_2O, and 0.5 μl of the *Sph*I restriction enzyme (New England Biolabs, Inc., Beverly, MA, USA) for the restriction fragment length polymorphism (RFLP) analysis. Each digestion reaction was incubated at 37°C for 6 h and analyzed with 2% agarose gel electrophoresis.

Statistical analysis

Group differences were analyzed using chi-squared statistics. Differences were considered significant when $P < 0.05$. The Hardy–Weinberg equilibrium was tested in the control groups to rule out sampling bias. The

Table 1 Clinicopathological parameters

	Control	*OSCC*	*OSF*
n	191	192	73
Age (mean ± SD) (range)	47.04 ± 8.98 (28–82)	51.84 ± 9.71 (30–75)	40.04 ± 11.66 (19–65)
Site			
BM		102	
Tongue		44	
Gingiva		32	
Others		14	
Stage and TNM			
Stage I–III		85	
Stage IV		107	
$n = 0$		122	
$n > 0$		70	
T1–3		94	
T4		98	

OSCC, oral squamous cell carcinoma; OSF, oral submucosal fibrosis; BM, buccal mucosa.

survival analysis was performed with the Kaplan–Meier survival curves and the log rank test.

Results

Restriction fragment length polymorphism analysis following *Sph*I digestion indicated only one band in subjects with C/C genotype, two bands in those with T/T genotype, and three bands (a 435-, 247-, and 188-bp band) in those with C/T genotype. The accuracy of the RFLP pattern was confirmed by direct sequencing (Fig. 1). For the controls, the allelic distribution of *MMP-9* was in the Hardy–Weinberg equilibrium. The T allele frequencies in control, OSCC, and OSF subjects were 0.14, 0.14, and 0.16, respectively. For the allelic distribution of the *MMP-9* −1562 C > T polymorphism, no significant difference was found between OSCC and control subjects or between OSF and control subjects (Table 2), indicating that the *MMP-9* −1562 C > T polymorphism was not associated with risks for either OSCC or OSF. The analysis performed on OSCC subsets stratified according to the lesion sites, metastasis, invasion, and stage also indicated that this polymorphism was not related to any clinicopathological parameter. No significant difference in the survival of OSCC patients was noted between different MMP-9 genotypes.

Transcriptional activity is higher for the *MMP-3* promoter containing 5A allele than the one containing 6A allele (31). A recent study demonstrated the co-effect of functional *MMP-3* −1171 5A > 6A and *MMP-9* −1562 C > T polymorphisms on atherosclerosis (32). Our previous study showed that *MMP-3* −1171 5A > 6A polymorphism is a risk factor for OSF, but not for OSCC (33). We further analyzed whether *MMP-9* and *MMP-3* polymorphisms could jointly influence the risk of OSCC and found no such influence on either the risk or prognosis of OSCC.

Investigation of age-related change in the MMP-9 expression in cultured aortic smooth muscle cells (SMC) has shown that MMP-9 expression is reduced in aged SMC (34, 35). After the OSCC sample was stratified by the median age, an association of *MMP-9* −1562 C > T polymorphism with the risk of OSCC was demonstrated in younger patients (aged < 51; $P = 0.029$; Table 3). Interestingly, in the older OSCC subset, the T allelic frequency was significantly higher in patients with BM OSCC than in patients with non-BM OSCC (0.21 vs. 0.08; $P = 0.027$) (Table 4).

Discussion

Only male areca chewers were chosen as subjects in this study to exclude the effect of gender. MMP-9 over-

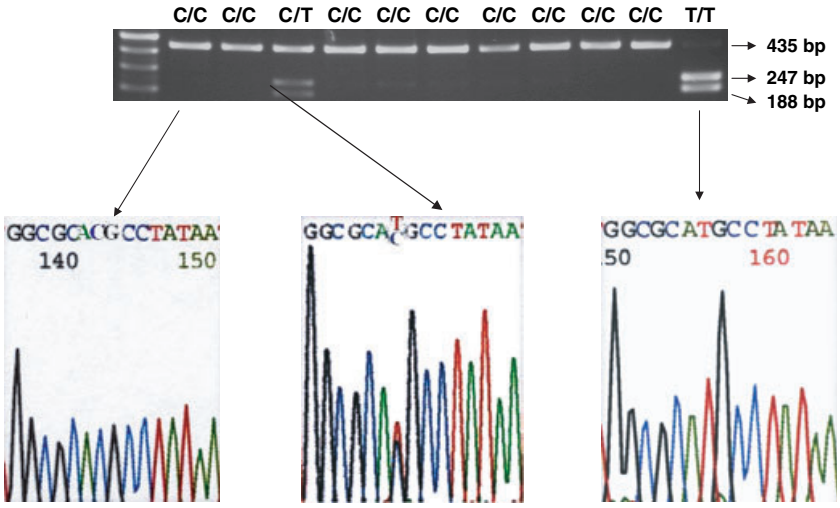

Figure 1 Restriction fragment length polymorphism (RFLP) analysis of *MMP-9* −1562 C > T polymorphism. The T/T genotype yields two distinguished bands (247 and 188 bp), where C/C genotype yields only one band (435 bp). The heterozygous C/T genotype yields three bands (upper panel). RFLP results were validated by direct sequencing analysis (lower panel).

Table 2 *Matrixmetalloproteinase-9* genotypings

	n	*T/T Allelotype*	*C/T Allelotype*	*C/C Allelotype*	*T Genotypic frequency*	*P-value*
Control	191	1	50	140	0.14	
OSCC	192	5	43	144	0.14	0.200
OSF	73	0	24	49	0.16	0.471

Chi-squared analysis.
OSCC, oral squamous cell carcinoma; OSF, oral submucosal fibrosis.

Table 3 Association between *MMP-9* −1562C > T polymorphism and OSCC risk in various age groups

Age (years old)	*T/T allelotype %*	*C/T allelotype %*	*C/C allelotype %*	*T genotypic frequency*	*P-value*
< 51					
Control	0.0	26.0	74.0	0.13	
OSCC	4.5	18.0	77.5	0.14	0.029
OSF	0.0	33.3	66.7	0.17	0.374
≥51					
Control	1.5	26.5	72.0	0.15	
OSCC	1.0	26.2	72.8	0.14	0.955
OSF	0.0	31.3	68.7	0.16	0.834

Chi-squared analysis.
OSCC, oral squamous cell carcinoma; OSF, oral submucosal fibrosis.

Table 4 Association between *MMP-9* −1562C > T polymorphism and clinicopathological parameters in various age group

Age (years old)	*T/T allelotype %*	*C/T allelotype %*	*C/C allelotype %*	*T genotypic frequency*	*P-value*
< 51					
BM	3.6	12.7	83.7	0.10	0.304
Non-BM	6.1	24.2	69.7	0.18	
$n = 0$	4.8	17.7	77.5	0.13	0.970
$n > 0$	3.7	18.5	77.8	0.14	
Stage I–III	2.2	15.6	82.2	0.10	0.449
Stage IV	6.8	20.5	72.7	0.17	
T1–3	2.0	18.0	80.0	0.11	0.434
T4	7.7	18.0	74.3	0.17	
≥51					
BM	2.1	38.3	59.6	0.21	0.027
Non-BM	0.0	17.5	82.5	0.09	
$n = 0$	1.7	25.0	73.3	0.14	0.669
$n > 0$	0.0	27.9	72.1	0.14	
Stage I–III	0.0	27.5	72.5	0.14	0.713
Stage IV	1.6	25.4	73.0	0.14	
T1–3	0.0	25.0	75.0	0.13	0.657
T4	1.7	27.1	71.2	0.15	

Chi-squared analysis.
BM, buccal mucosa; OSCC, oral squamous cell carcinoma; OSF, oral submucosal fibrosis.

expression was observed in head and neck carcinomas and this was closely related to invasion, metastasis, and p53 mutation (21, 36, 37). The findings suggest that MMP-9 plays important roles in head and neck carcinogenesis. Although functional *MMP-9* −1562 C > T polymorphism was reported to influence the transcriptional activity of *MMP-9* and was associated with susceptibility to and prognosis in many diseases, our results do not substantiate a strong link between this polymorphism and the risk of areca-associated OSCC. The risk of OSCC was significant in only younger male areca chewers bearing the T allele. The impact of age on *MMP-9* expression could be explained by the fact that in older individuals, activities of NF-κB and AP-1 transcription factors can be lower and thereby reduce the basal *MMP-9* promoter activity (34, 35). Thus, change in *MMP-9* transcriptional activity secondary to genomic polymorphism would be minimized.

Previous studies relating functional MMPs promoter polymorphisms to the risk or pathogenesis of areca-associated oral diseases found an association of *MMP-1* and *MMP-2* with the OSCC risk and an association of *MMP-3* with OSF risk (30, 33). Human *MMP*-1, 3, 7, 8, 10, 12, 13, 20, and 27 genes are clustered on chromosome 11q; whereas *MMP-2* locus maps to 16q13 and *MMP-9* to 20q11. The involvement of MMP-3 and MMP-9 in invasion and metastasis was shown during the progression stage of oral carcinogenesis (33, 37, 38). Since linkage disequilibrium might complicate haplotypic effects of multiple gene polymorphisms on the risk of the disease (30), a cross analysis using previous *MMP-3* data (33) and data from the present study were performed. The analysis indicated that functional *MMP-3* and *MMP-9* polymorphisms have no joint effects on survival or OSCC risk. From our data and the importance of MMP for tumor progression, we conclude that the haplotype of *MMP-3* and *MMP-9* polymorphism is probably irrelevant to the initiation of oral carcinogenesis. A recent study revealed that the functional *MMP-9* polymorphism does not affect the level of circulating MMP-9 in healthy subjects (39), but plasma MMP-9 activity was elevated in patients with coronary artery disease and head and neck cancer (40, 41). Since the circulating MMP-9 level and functional

MMP-9 polymorphism were also risk factors for coronary artery disease and prognostic factors for gastric cancers (3, 19, 33, 42, 43), it was postulated that the effect of *MMP-9* polymorphism would become more apparent when the gene is aberrantly induced in various disease states including carcinogenesis (39).

Our results also showed a rather high T allele frequency in BM OSCC compared to non-BM counterpart OSCC in older subjects. The non-keratinizing squamous epithelium on the BM surface is the most vulnerable target for the damaging effects of areca. Most of our areca chewers were in the BM OSCC subset. The disproportionate prevalence of BM OSCC among areca chewers might be explained by the fact that areca can induce MMP-9 expression and activity in saliva (44) and the induction might involve the enzyme encoded by the T allele. The present study provided additional evidence for the hypothesis that BM OSCC in areca chewers is a specific entity distinct from non-BM OSCC in areca chewers (3, 30, 44, 45).

This study found no strong correlation of the functional *MMP-9* −1562 C > T polymorphism in the *MMP-9* promoter with the risk of either OSCC or OSF in male areca chewers. However, this correlation became significant when the study cohort was restricted to younger subjects. Because the role of the −1562 C > T polymorphism might be influenced by age and other factors, these interactions should be further verified.

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