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Functional genotype in *matrix metalloproteinases-2* promoter is a risk factor for oral carcinogenesis

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BACKGROUND: *Matrix metalloproteinase-2* (*MMP-2*) can degrade extracellular matrix and basement membrane, and play an important role in the development and progression of multiple carcinomas, including oral squamous cell carcinoma (OSCC). A $-1306C \rightarrow T$ polymorphism in the *MMP-2* promoter disrupts Sp1-binding site, and results in reduction of transcriptional activity. This study aimed to assess the association of such genotype with the risk of OSCC and oral submucous fibrosis (OSF), which is a precancerous condition that exhibits excessive collagen production and etiologically links to areca use.

METHODS: Genomic DNA from the blood samples of 121 OSCC cases, 58 OSF cases and 147 controls were amplified by polymerase chain reaction (PCR) and subjected to denaturing high-performance liquid chromatography (dHPLC) analysis for genotyping. The OSCC were further classified into buccal squamous cell carcinoma (BSCC) and non-buccal squamous cell carcinoma (NBSCC), according to the site of involvement. Fisher's exact test and unconditional logistic regression models were used for statistical analysis.

RESULTS: Subjects carrying *CC* genotype had nearly twofold increased risk for developing OSCC when comparing with *CT* or *TT* genotype. Subjects carrying *CC* genotype had more apparent risk (greater than fourfold) for developing NBSCC. However, no increase in risk for lymph node metastasis or advanced stage was identified in OSCC cases carrying such genotype. Preliminarily data suggest no significant association between subjects carrying *CC* genotype and the development of BSCC or OSF.

CONCLUSION: This is the first paper demonstrating that functional genotype of *MMP-2* promoter is a risk factor for oral carcinogenesis, particularly for the subsets occurring on non-buccal site.

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Matrix metalloproteinases (MMPs) is a superfamily of proteolytic enzymes capable of degrading extracellular matrix and basement membrane (1). Overexpression of MMPs in multiple carcinomas was found to be important for tumor invasion and metastasis (2). Recent studies have also indicated that MMPs are involved in early tumorigenesis, modulating proliferation, apoptosis and angiogenesis. *MMP-2* primarily hydrolyzes type IV collagen, which is a key element of basement membrane (1, 2). In addition, MMPs can cleave growth factors in extracellular matrix (2). For instance, *MMP-2* can degrade insulin-like growth factor-binding proteins and release insulin-like growth factors, which modulate proliferation and apoptosis of cells (2, 3). Thereby, *MMP-2* activities might be highly involved in tumorigenesis and tumor progression.

The *MMP-2* promoter contains sequences for the binding of AP-2, p53, Sp1, and Sp3. Price et al. (4) identified a $-1306C \rightarrow T$ polymorphism in *MMP-2* promoter. This base transition located in CCACC box of Sp1-binding site and eliminates the promoter activity (4). It is likely that *CC* genotype may be associated with the high transcriptional and enzyme activity of *MMP-2*, and eventually affect individual susceptibility to neoplasms. Yu et al. (5) have shown that the frequency of the *CC* genotype was significantly higher in lung carcinoma cases when compared with controls. Recently, Miao et al. (3) have identified that *CC* genotype is associated with the risk of a subset of gastric adenocarcinoma, localized on cardia. The global role of functional genotype of *MMP-2* for the risk of various neoplasms needs to be studied.

Overexpression of *MMP-2* is a frequent event in OSCC (6). *MMP-2* plays critical roles in invasion and metastasis of OSCC (7–10). Expression of *MMP-2* predicts poor prognosis in OSCC including tongue carcinomas (11, 12). OSCC, particularly the

subset – buccal squamous cell carcinoma (BSCC) is prevalent in most Asian countries because of the unique habit of areca chewing (13–19). *MMP-2* is also overexpressed in areca-associated OSCC (20). Oral submucous fibrosis (OSF) is a disease, occurring exclusively in areca chewers (15). It is characterized by disturbances in the balance occurring between synthesis and degradation of extracellular matrix including collagen. A recent study has reported that arecoline, a key areca ingredient, inhibits the gelatinolytic activity of *MMP-2* (21). Thereby, *MMP-2* genotype may be associated with the susceptibility of OSF.

The present case–control study aims to investigate the contribution of the −1306*CC* genotype in *MMP-2* promoter to risk for OSCC and OSF.

Materials and methods

Samples

A total of 121 OSCC and 58 OSF cases were obtained from Oral and Maxillofacial Surgery Department at Taipei Mackay Memorial Hospital. Of which 147 control subjects were selected from people who came for routine physical checkups, and had non-neoplastic minor oral operations or maxillofacial trauma in the same hospital. Those with autoimmune disorders, blood diseases and previous malignancy were excluded from the control group. The clinical parameters of OSCC and OSF cases, including age, gender and areca use, are described in Table 1. The OSCC cases were further grouped on the basis of site, lymph node metastasis (LNM) and clinical stage (Table 1). For instance, the OSCC was subgrouped as BSCC and non-buccal squamous cell carcinoma (NBSCC). This study was approved by an ethics reviewing committee. Blood was drawn from the subjects. A leukocyte cell pellet was obtained from the buffy coat by centrifugating whole blood. DNA was isolated by Blood Mini Kit (Qiagen, Valencia, CA, USA).

MMP-2 genotyping

The 295-bp sequence of *MMP-2* promoter region was amplified by polymerase chain reaction (PCR) using the sense primer: 5′-CTGACCCCCAGTCC TATCTGCC-3′ and antisense primer 5′-TGTTGG GAACGCCTGACTTCAG-3′ (5). The PCR reaction mixture (25 μl) contained 20 ng genomic DNA, 0.2 mM dNTP, 1.0 unit Prozyme DNA polymerase (Protech Enterprise, Taipei, Taiwan) with 1X PCR buffer. The reaction was conducted under the following conditions: an initial melting step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 64°C, and 45 s at 72°C; and a final elongation of 10 min at 72°C. Denaturing high-performance liquid chromatography (dHPLC) analysis was performed on a Transgenomic WAVE System (Transgenomic Inc., Omaha, NE, USA) following methods described (5). Each amplicon was applied to the dDHPLC column, denatured for 1 min at 94°C, and then gradually re-annealed by decreasing sample temperature from 94 to 45°C over a period of 30 min to form homo- and/or heteroduplexes. PCR products were eluted by a linear acetonitrile gradient at the flow rate of 0.9 ml/min.

Table 1 Clinical parameters of oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSF) cases

	OSCC	*OSF*
n	121	58
Age (mean ± SE)	51.3 ± 0.9	39.1 ± 1.4
Gender (male/female)	115/6	58/0
Areca use	97	58
Site		
Non-buccal mucosa	49	–
Buccal mucosa	72	–
LNM		
0	77	–
>0	44	–
Stage		
I–III	64	–
IV	57	–

LNM, lymph node metastasis.

DNA sequencing

Direct sequencing of gel-purified amplicons (Qiaex II Gel Extraction Kit; Qiagen) was performed using a 377-18 DNA sequencer (Applied Biosystem, Foster City, CA, USA) and sense primer to confirm in selected cases the genotypes revealed by dHPLC.

Statistical analysis

Evaluation of the Hardy–Weinberg equilibrium was performed by comparing observed and expected genotypes, using the chi-square analysis. Fisher's exact test was used to examine differences between controls and cases. The associations between the *MMP-2* genotype and risk of disease genesis was estimated by odds ratio (OR) and associated 95% confidence interval (CI), which were calculated by unconditional logistic regression models.

Results

The genotyping of *MMP-2* using dHPLC system revealed the double peak elution pattern of heterozygous *CT* genotype (Fig. 1a). The homozygous genotypes were determined by the single peak elution pattern in the first dHPLC (Fig. 1b). A second dHPLC was performed on homozygous samples mixed with a known sequencing-confirmed reference (homozygous *CC* genotype) sample to generate heterozygous elution pattern. By this means, the homozygous *TT* genotypes can be further determined, as the mixture of this genotype with reference can produce heterozygous elution pattern that were readily discernable. Selected samples were sequenced directly to confirm the results of dHPLC (Fig. 1c,d).

The distribution of *MMP-2* genotypes in controls was in Hardy–Weinberg equilibrium, i.e. the observed and expected figures did not differ. Three *MMP-2* genotypes of controls were 73%, 23% and 4% for *CC, CT* and *TT* genotype, respectively. The frequencies for *C* genotype of the controls and OSCC cases were 0.84 and 0.92, respectively (Table 2). None of the OSCC cases carried

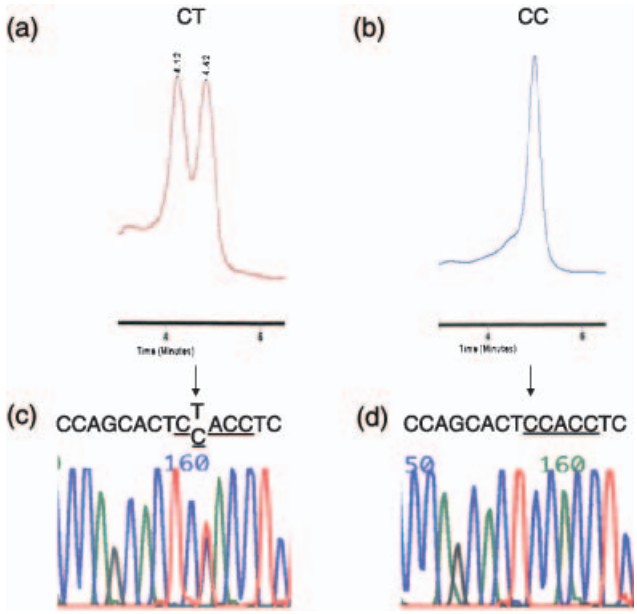

Figure 1 Genotyping and sequencing. Representative elution patterns of high-performance liquid chromatography (dHPLC) for heterozygous genotype in (a) and homozygous genotype in (b). Direct sequencing in (c) and (d) confirms the *CT* genotype and *CC* genotype in (a) and (b), respectively.

TT genotype, whereas, 4% of the controls carrying this genotype. As *TT* genotype was rare, it was combined with the *CT* genotype as the variant group for subsequent estimation of risk, using logistic regression analysis. The frequency of *CC* genotype was significantly higher in OSCC cases than that of the controls ($P = 0.04$, Table 2). The OR of OSCC for subjects carrying the *CC* genotype was 1.89 (95% CI: 1.03–3.45).

A significant higher frequency for *CC* genotype (0.92) was noted in NBSCC subset of OSCC cases when compared with the controls ($P = 0.005$, OR = 4.21, 95% CI: 1.42–12.45, Table 2). However, it was not the case for BSCC subset of OSCC cases. There was a significant difference in genotypic distribution between NBSCC and BSCC ($P < 0.05$). No OSF cases carry *TT* genotype and the frequency of C genotype (0.91) in OSF cases was highly similar to the situation in OSCC cases. However, no statistically significant difference was observed between OSF cases and control cases (Table 2).

The frequency of *CC* genotype showed no significant difference between cases with and without LNM (0.86 vs. 0.82; $P = 0.62$). The OR was 1.41 (95% CI: 0.60–3.97) for the *CC* genotype compared with variant genotypes. Difference in *CC* genotype was not observed in OSCC cases with variable age, areca use history and clinical stage (detailed analysis not shown).

Discussion

The *MMP-2* plays an important role in multiple stage carcinogenesis. A number of studies have shown that *MMP-2* is overexpressed in various cancer tissues and its involvement in tumor initiation, invasion, angiogenesis and metastasis was critical (2–5). In OSCC, the gelatinolytic activities of *MMP-2* in most cancer cell nests were much higher than those of adjacent normal oral epithelium (8). The $-1306C \rightarrow T$ transition in the promoter region of *MMP-2*, which disrupts an Sp1-binding site, can lead to a remarkable lower promoter activity (4). The presence of Sp1 consensus sequence at *MMP-2* −1306*C* nucleotide may enhance transcription, which produces higher levels of *MMP-2* protein in subjects carrying the *CC* genotype than those carrying the variants. It is reasonable to assume that subjects carrying germ-line *CC* genotype would have increased expression of this enzyme for long period and they may be more susceptible to cancer. We found that subjects carrying the *CC* genotype had a approximately twofolds increased risk for developing OSCC and the risk for NBSCC was even higher (greater than fourfolds). The *2G* genotype, which causes a functional polymorphism in *MMP-1* promoter, has also been identified as a risk factor for OSCC in our previous study (18). The synergistic functional genotype in MMPs might be very useful in early identification of OSCC risks.

Overexpression of *MMP-2* has been shown to be correlated to the invasion and metastasis of multiple cancers, including OSCC (2, 7, 8, 10–12). The activities of *MMP-2* in metastatic OSCC cells were significantly higher than those of non-metastatic counterparts. Thereby, the *MMP-2* overexpression or activity has been considered as a marker to predict tumor progression (8). Whereas, evidences from us indicated such genotype had no impact on tumor progression, reflecting by metastasis or advances in clinical stage. It argues the role of the *CC* genotype as a relevant genetic factor, which induces the local overexpression

Table 2 The risk of *matrix metalloproteinase-2* (*MMP-2*) genotype for oral diseases

	n	*C genotype frequency*	*CC (%)*	*CT (%)*	*TT (%)*	*P-value*[a]	*OR*	*95% CI*
Control	147	0.84	107 (73)	34 (23)	6 (4)		1	Referent
OSCC	121	0.92	101 (83)	20 (17)	0 (0)	0.04	1.89	1.03–3.45
NBSCC	49	0.95	45 (92)	4 (8)	0 (0)	0.005	4.21	1.42–12.45
BSCC	72	0.89	56 (78)	16 (22)	0 (0)	0.51	1.31	0.67–2.54
OSF	58	0.91	47 (81)	11 (19)	0 (0)	0.28	1.60	0.75–3.38

[a]*CC* vs. *CT* and *TT*.
OSCC, oral squamous cell carcinoma; NBSCC, non-buccal squamous cell carcinoma; BSCC, buccal squamous cell carcinoma; OSF, oral submucous fibrosis; OR, odds ratio; CI, confidence interval.

of *MMP-2*, and as a risk marker of tumor dissemination. This could be partially interpreted by the fact that multiple MMPs are involved in invasion or metastasis of tumors that might mask the crucial role of *MMP-2* genotype (1, 2). Additional surveys using more samples and confounders, such as survival, are required to further insight the importance of *MMP-2* in OSCC progression.

The contradictory findings in the association between *CC* genotypes and the risk of NBSCC or BSCC were interesting. The data suggest that the *MMP-2* genotype might have profound effect on the risk of getting carcinomas at different locations in oral cavity. BSCC is the most common subset of OSCC in areca chewing populations, which accounts for more than a half of the cases, but it is extremely rare in the West (13–17, 19). Previous studies from us have specified the great molecular discrepancies between NBSCC and BSCC in *p16/MTS1* mutation and loss of RAR-β mRNA expression (16, 19). The frequency of a functional AA genotype in *CCND1*, which constitutes a risk factor for oncogenesis, was also contradictory between NBSCC and BSCC (17). Recently, we have also shown that functional *2G* genotype in *MMP-1* gene was a risk confounder for NBSCC, but not for BSCC (18). In present study, we found that the *C* genotype frequency of *MMP-2* in BSCC (0.89) and controls (0.84) was quite similar, which might lead to a speculation that specific factors may underlie the malignant transformation of BSCC, which bypass or counteract with the demands of functional *MMP-2* genotype. Evidences accumulated so far might suggest that BSCC, the major OSCC subset tightly associated with areca use in Asian, might undertake distinctive pathways for tumorigenesis.

The disruption in collagen synthesis and the degradation of collagen as a result of areca chewing are pathogenetic features of OSF. Most recent evidences have linked the *MMP-2* activity in fibroblast as an event affected by areca ingredients (21). Our analysis indicated a lack of association between *MMP-2*genotype and OSF risk. However, the *C* genotype frequency of OSF (0.91) is indifferent from that of OSCC (0.92), and far higher than controls. It is still likely that *MMP-2* genotype might be associated with the susceptibility of OSF, if the sample size of future study can be increased. As multiple cytokines and growth factors associated with collagen production, play roles in the pathogenesis of OSF (15), the potential of functional genotype in other MMPs as risk factors of OSF remained to be elucidated.

In conclusion, the present study provides evidences for the first time that $-1306C \rightarrow T$ polymorphism in *MMP-2* promoter is a susceptibility factor for the development of OSCC, with the *CC* genotype being associated with the increase of risk. This association is especially worth noting in NBSCC subset of tumors.

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