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Correlation between functional genotypes in the *matrix metalloproteinases-1* promoter and risk of oral squamous cell carcinomas

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BACKGROUND: Oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSF), which are highly associated with areca use, are prevalent in most Asian countries. Matrix metalloproteinases (MMPs) are superfamily of metal-dependent proteolytic enzymes, mediating the degradation of extracellular matrix. Insertion/deletion (–1607 2G → 1G) polymorphism has been described in the promoter region of the human matrix metalloproteinases-1 (*MMP-1*) genes, which cause an alteration in the transcriptional activity. This genotype is associated with risks of cancer genesis and metastasis. In this paper, we studied the relationship between such genotype and areca-associated oral diseases.

METHODS: Genomic DNA from the blood of OSCC ($n = 121$), OSF ($n = 58$) cases and controls ($n = 147$) were amplified by polymerase chain reaction (PCR)-based genotyping. The OSCC were further grouped into buccal squamous cell carcinoma (BSCC) and non-buccal suqmaous cell carcinoma (NBSCC), in accord with the site of involvement. The significance of the differences was assessed by Fisher's exact test.

RESULTS: The 2G genotype in *MMP-1* promoter was observed with a higher frequency in both OSCC (0.69, $P = 0.06$) and NBSCC (0.76, $P = 0.03$) cases compared with controls (0.63), with an odds ratio of 2.17 and 4.58, respectively. This genotype was not related to the risk of OSF. No other clinicopathologic parameter was associated with the genotypes in OSCC cases.

CONCLUSION: The results showed that 2G genotype in *MMP-1* promoter was associated with the risk of OSCC.
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Oral squamous cell carcinoma (OSCC) has been the most common malignancy in areca-using region, accounting for up to 50% of malignant tumors in some south Asian countries. It is also a prevalent disease in Taiwan as the fifth leading malignancy in male population. Moreover, areca chewing is highly associated with oral submucous fibrosis (OSF) and variable leukoplakias, which are pre-cancerous condition or lesions (1–6). Carcinogenesis of OSCC involves the alterations in cellular proliferation, apoptosis and migration that are intimately linked to the abnormalities in molecular regulation machinery. The absence of normal regulation of such genes may advance tumorigenesis.

The matrix metalloproteinases (MMPs) constitute a superfamily of at least 24 human metal-dependent proteolytic enzymes, which degrade extracellular matrix and basement membrane. MMPs are synthesized and secreted by cancer cells and by adjacent stromal cells (7, 8). Therefore, it is believed that the MMPs, via breaking down of the physical barrier, play a pivotal role in tumor invasion and metastasis (8, 9). In addition, recent studies indicated that MMPs were also important in the neoplastic process of malignancies (10). Functional nucleotide polymorphisms in genes, which regulate neoplastic process, cell migration and cancer invasion, are good candidates for investigation of genetic susceptibility.

Matrix metalloproteinases-1 (*MMP-1*) is an interstitial collagenase acting on fibrillar collagen and gelatin. A functional polymorphism in the promoter region of human *MMP-1* gene has been found (11). Two alleles, 2G and 1G, were formed by an insertion/deletion of a guanine at position 1607. The 2G genotype has been shown to significantly increase the transcriptional activity when it was compared with 1G genotype through the creating of ETS (erythroblast transformation specific) (5′-GGA-3′) binding site (11). Thereby, the increase of 2G genotype has been associated with the risks of carcinomas of ovary (12), lung (13) and colon-rectum (14, 15), and the invasion of colorectal carcinomas (15).

The overexpression of *MMP-1* is a frequent event in OSCC (16, 17). However, the involvement of functional *MMP-1* genotype in the development and progression of OSCC has not been addressed yet. The aim of this study was to investigate possible correlations between the genotype of *MMP-1* promoter in OSCC and OSF to specify the genetic susceptibility.

Materials and methods

Subjects

A total of 121 OSCC and 58 OSF cases were obtained from Oral and Maxillofacial Surgery Department at Taipei Mackay Memorial Hospital. One hundred and forty-seven 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 basic clinical parameters of OSCC and OSF subjects, including age, gender, and areca use, are described in Table 1. The OSCC cases were further grouped on the basis of site (buccal mucosa vs. non-buccal mucosa), lymph node metastasis (LNM) (0 vs. >0) and clinical stage (I–III vs. IV) (Table 1). This study was approved by an ethics reviewing committee. After an informed consent was obtained, blood was drawn from the subjects. A leukocyte cell pellet was obtained from the buffy coat by centrifugating whole blood. DNA was isolated by Qiagen Blood Mini Kit (Qiagen, Valencia, CA, USA).

MMP-1 genotyping

−1607 2G/1G polymorphism in the *MMP-1* promoter was determined by polymerase chain reaction (PCR)-based genotyping method. The primers used to generate amplicons of 149-bp (for 2G), 148-bp (for 1G) or the mixture (for 2G/1G) were *MMP-1*-P-sense: 5′-GTTA-TGCCACTTAGATGAGG-3′ and *MMP-1*-P-anti-sense: 5′-TTCCTCCCCTTATGGATTCC-3′. The 5′-site of the sense primer was labeled with FAM fluorescence dye. The amplification reaction mixture (15 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 reaction was carried out in three steps: first, 2 min at 94°C; then, 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; lastly, 5 min 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 fluoresence 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 oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSF)

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

DNA sequencing

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

Statistical analysis

The variants were analyzed using Fisher's exact test. Differences between the values were considered significant when $P < 0.05$. The associations between the *MMP-1* genotype and risk of disease genesis were estimated by odds ratio (OR) and their 95% confidence interval (CI) were calculated by unconditional logistic regression models.

Results

The genotyping of *MMP-1* promoter was undergone by Genescan system. It distinguished homozygous 2G genotype (2G/2G), heterogenous 2G genotype (2G/1G) and homozygous 1G (1G/1G) genotype patterns on the basis of differential mobility of amplicons with different sizes (Fig. 1a). In the selected samples, homozygous 2G or homozygous 1G genotype were confirmed by direct sequencing of the amplicons (Fig. 1b).

The frequency for 2G genotype was 0.63 in controls (Table 2). There was a difference in the frequency for 2G genotypes lying between controls and OSCC cases (0.69) with a marginal statistical significance ($P = 0.06$, OR = 2.17, 95% CI = 0.99–4.73, Table 2). A significant higher frequency for 2G genotypes was noted in non-buccal squamous cell carcinoma (NBSCC) subset of OSCC cases (0.76) when compared with controls ($P = 0.03$, OR = 4.58, 95% CI = 1.04–20.17, Table 2). However, it is not the case for buccal squamous cell carcinoma (BSCC) subset of OSCC cases. BSCC and OSF were noted to have the genotypic distribution similar to the controls (Table 2).

The genotyping revealed no statistically significant difference in the frequency of 2G genotype in OSCC cases, which exhibit different LNM and clinical stage (detailed analysis not shown).

Discussion

The 2G/1G insertion/deletion polymorphism at −1607 in the promoter region of the *MMP-1* gene, which

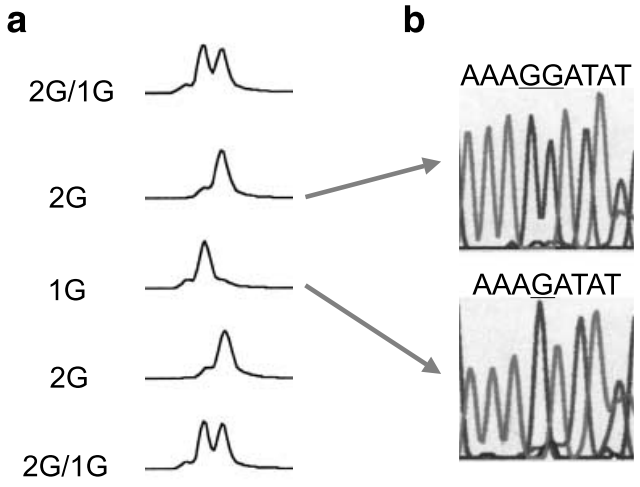

Figure 1 Genotyping and sequencing. (a) Representative genotyping profile of amplicons. *X*-axis, mobility of amplicons; *Y*-axis, intensity of fluorescence. Note the distinguishable separation of each peak. The left peak represents 1G genotype and the right peak represents 2G genotype. (b) Direct sequencing of homozygous 2G genotype and homozygous 1G genotype in selected samples.

determines the presence of an ETS-binding site, affects the transcriptional level of *MMP-1* in cells (11). It is conceivable that higher transcriptional activity of *MMP-1* associated with the 2G genotype may enhance tumor formation and invasiveness. Previous studies have supported this notion in multiple carcinomas (12–15). Furthermore, a correlation between the transcription-enhancing of 2G genotype and *MMP-1* overexpression has been identified in ovarian carcinomas (12). We demonstrated for the first time that 2G genotypes were more frequent in OSCC cases than they were in the controls. Although high frequency of homozygous 2G genotype might have been anticipated in tumor cells and tissues, we found that heterozygous 2G genotype were also associated with the increased risk of oral carcinogenesis which is consistent with the findings in ovarian carcinomas (12). This increase in frequency is statistically significant in NBSCC. It is important to note the rather wide CI value in spite of the good OR lying in the comparison between control and NBSCC. This might suggest the necessity to further confirm the risk of NBSCC and *MMP-1* genotype using a large amount of samples.

The contradictory findings for the association of 2G genotypes for the risk of NBSCC and the absence of association in BSCC were intriguing. The data suggest that the *MMP-1* genotype might have profound impact on the risk of carcinomas at different locations in the oral cavity. BSCC is the most common type OSCC in areca chewing populations, while it is extremely rare in the West (1–6). Previous studies from us have specified the exclusive occurrence of the *p16/MTS1* mutation in BSCC relative to NBSCC (1). BSCC exhibits significantly more loss of *RAR-β* expression in contrast to NBSCC (4). In addition, the frequency of homozygous A/A genotype in *CCND1*, which was considered to be a risk factor for oncogenesis, was also significantly lower in BSCC in relation to NBSCC (2). We found in the present study that similar distribution of *MMP-1* genotypes in BSCC and controls might lead to a speculation that specific factors may underlie the malignant transformation of BSCC, which bypasses or counteracts with the demands of functional *MMP-1* genotype as opposed to NBSCC. The evidences accumulated suggest that BSCC and NBSCC might undertake distinctive molecular pathways for tumorigenesis.

The OSF is a disease occurring exclusively in areca chewers (3, 5). It is considered to be an inflammatory situation in response to areca ingredients or physical irritation. It was reported that *TNF*-2 genotype, a high production variant, is significantly lower in OSF cases than in controls (6). Despite no evidence links the functional involvement of *MMP-1* to the pathogenesis of OSF, the excessive production and remodeling of collagen in OSF urged us to genotyping *MMP-1* for OSF cases. The results indicated a negative association between *MMP-1* genotype and OSF risk. All BSCC cases in our study cohort are areca chewers. Both OSF cases and BSCC cases had the genotypic distribution rather similar to the controls (Table 2). Such findings preliminarily exclude the involvement of *MMP-1* genotype in areca-associated oral diseases. However, multiple cytokines and growth factors play roles in the synthesis and deposition of collagen in OSF (3), the functions of other MMPs as pathogenetic factors remained to be elucidated. Our preliminarily analysis, which identified that lymph node involvement of OSCC did not correlate to *MMP-1* genotype, were in agreement with findings in colorectal carcinomas (14). This scenario could be partially explained by the high fraction of BSCC, which might skip the need of *MMP-1* for tumor progression, is our study cohort. In future, a clinical study should be undertaken in a larger population to investigate the correlation between *MMP-1* genotype and progression of NBSCC.

Table 2 Genotypes in matrix metalloproteinases-1 (*MMP*-1) promoter related to oral diseases

	n	*2G genotype frequency*	*2G/2G*	*2G/1G*	*1G/1G*	*P-value**	*OR**	*95% CI*
Control	147	0.63	63 (43)	60 (41)	24 (16)		1	Referent
OSCC	121	0.69	57 (47)	54 (45)	10 (8)	0.06	2.17	0.99–4.73
NBSCC	49	0.76	27 (55)	20 (41)	2 (4)	0.03	4.58	1.04–20.17
BSCC	72	0.65	30 (42)	34 (47)	8 (11)	0.42	1.56	0.67–3.67
OSF	58	0.67	27 (46)	24 (52)	7 (12)	0.52	1.42	0.58–3.51

*2G/2G and 2G/1G vs. 1G/1G; OR, odds ratio; CI, confidence interval (%); OSCC, oral squamous cell carcinoma; NBSCC, non-buccal squamous cell carcinoma; BSCC, buccal squamous cell carcinoma; OSF, oral submucous fibrosis.

In conclusion, our results indicated that 2G genotype in *MMP-1* promoter could be a risk factor for oral carcinogenesis, especially for those cases which did not occur on buccal mucosa. The genotyping of other MMPs as potential markers for susceptibility to OSCC allows a precise and early identification of individuals at high risk. In addition to prediction and prevention, the findings will aid the design of therapeutic modalities and evaluation of treatment outcome.

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