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Contribution of *MMP2* Promoter Genotypes to Oral Cancer Susceptibility, Recurrence and Metastasis in Taiwan

CHIA-WEN TSAI[1*], HUAI-MEI HSU[1,2*], YUN-CHI WANG[1*], WEN-SHIN CHANG[1,2], LIANG-CHUN SHIH[3], KUO-TING SUN[4], YI-WEN HUNG[5], YI-CHIN YANG[5], CHI-LI GONG[6] and DA-TIAN BAU[1,2,7]

[1]Terry Fox Cancer Research Laboratory, Translational Medicine Research Center,
[3]Department of Otolaryngology, and [4]Division of Pediatric Dentistry,
China Medical University Hospital, Taichung, Taiwan, R.O.C.;
[2]Graduate Institute of Biomedical Sciences and [6]Department of Physiology,
China Medical University, Taichung, Taiwan, R.O.C.;
[5]Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan, R.O.C.;
[7]Department of Bioinformatics and Medical Engineering, Asia University, Taichung, Taiwan, R.O.C.

Abstract. *Aim: Metalloproteinase 2 (MMP2) is a multi-functional protein which has been shown to be up-regulated in patients with oral cancer, especially those with lymph node metastasis. However, the association of MMP2 genotype with oral cancer risk or metastatic behavior is unknown. This study aimed to evaluate the role of MMP2 promoter 1306 and -735 genotypes in the risk of oral cancer and metastasis. Materials and Methods: In this case–control study, MMP2 promoter 1306 (rs243865) and -735 (rs2285053) genotypes and their interaction with consumption of areca, cigarettes, and alcohol in determining oral cancer risk were investigated among 788 patients with oral cancer and 956 gender-matched healthy controls. In addition, their role in oral cancer metastasis were also examined. Results: The distribution of CC, CT and TT for MMP2 promoter 1306 genotype was 79.0, 20.1 and 0.9% in the oral cancer group and 68.7, 29.2 and 2.1% in the non-cancer control group, respectively (p for trend=4.3E-6). The allelic frequency distributions showed that the variant T allele of MMP2 promoter 1306 conferred lower oral cancer susceptibility than the wild-type C allele (odds ratio=0.61, 95% confidence interval=0.50-0.75, p=1.1E-6). As for the MMP2 -735 genotypes, there was no differential distribution in genotypic or allelic frequencies. The variant CT and TT genotypes were also associated with lower metastasis rates within 5 years among the patients with oral cancer (odds ratio=0.34, 95% confidence interval=0.15-0.80, p=0.0102). Conclusion: The CT and TT genotypes of MMP2 promoter 1306 may have a protective effect on oral cancer susceptibility and metastasis risk within 5 years for Taiwanese. They may serve as predictive markers for oral cancer in precise medical practice.*

From the viewpoint of epidemiology, oral cancer is the tenth most commonly diagnosed cancer worldwide, with the highest incidence density in Taiwan (1). According to the updated annual report from Taiwan government, oral cancer is the fourth cause of cancer-related death among males in Taiwan and fifth among all citizens (2). Unluckily, patients with oral cancer in Taiwan and all over the world suffer from the threat of recurrence and metastasis. Those at higher risk of oral cancer recurrence or metastasis should be detected earlier and followed-up more frequently to enjoy longer life, with the development of useful markers for prognosis prediction. Although several predictive biomarkers for oral cancer in Taiwan have been revealed (3-9), genomic biomarkers of oral cancer risk, especially those useful for prediction of recurrence/metastasis are of great interest. Among them, the practical biomarkers for oral cancer metastasis are urgently in need.

Extracellular matrix (ECM) structures play an important role in micro-environmental remodeling during tumorigenesis (10). The matrix metalloproteinases (MMPs) are a family of enzymes involved in ECM remodeling *via* controlling the degradation of ECM components, such as those in connective tissue matrices (10, 11). In literature, MMPs were reported to be related to the regulation of oral cancer invasion and metastasis (12).

*These Authors contributed equally to this study.

Correspondence to: Da-Tian Bau, Chi-Li Gong, Terry Fox Cancer Research Laboratory, Translational Medicine Research Center, China Medical University Hospital, 2 Yuh-Der Road, Taichung, 404 Taiwan, R.O.C. Tel: +886 422053366 (Ext. 5805), e-mail: datian@mail.cmuh.org.tw; artbau2@gmail.com

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Table I. *Characteristics of the 788 patients with oral cancer and 956 controls investigated.*

Characteristic	Controls (n=956)	Cases (n=788)	*p*-Value[a]
Age (years)			
Mean (SD)	56.6 (8.7)	55.8 (9.9)	0.7951
Gender, n (%)			
Male	727 (76.0%)	599 (76.0%)	>0.99
Female	229 (24.0%)	189 (24.0%)	
Personal habits, n (%)			
Areca chewing	506 (52.9%)	661 (83.9%)	**<0.0001**
Cigarette smoking	667 (69.8%)	595 (75.5%)	**0.0084**
Alcohol drinking	641 (67.1%)	560 (71.1%)	0.0773
Primary tumor site, n (%)			
Tongue		325 (41.2%)	
Buccal mucosa		294 (37.3%)	
Mouth floor		30 (3.8%)	
Retromolar trigone		26 (3.3%)	
Alveolar ridge		18 (2.3%)	
Palate		18 (2.3%)	
Lip		39 (4.9%)	
Other		38 (4.9%)	

SD: Standard deviation. [a]Based on Chi-square test. Statistically significant values are shown in bold.

In recent years, the role of MMPs in the process of tumor invasion has received continuous attention and it was reported that MMP2 played an important role in the degradation of extracellular matrix mediated by glioma cells (13). *MMP2* gene is located on chromosome 16q21 and composed of 12 introns and 13 exons (14). Promoter 1306 (rs243865) and -735 (rs2285053) single nucleotide polymorphisms (SNP) of *MMP2*, together with those of its inhibitor *TIMP2*, can affect their protein or mRNA expression and tumor invasion by altering the transcriptional activity of its own genes, eventually involving in the development of several types of cancer, including breast, lung, esophageal and colon cancer (15-18). MMP2 was reported to be up-regulated in patients with oral squamous cell carcinoma, especially those with lymph node metastasis (19). Thus, in the present work, a case–control genotyping study was performed to investigate the correlations of *MMP2* promoter 1306 (rs243865) and -735 (rs2285053) polymorphisms with the susceptibility and metastatic prognosis of oral cancer in Taiwan.

Materials and Methods

Oral cancer patient and control group collection. The current study was approved by the Institutional Review Board (DMR101-IRB1-306) of our Hospital. Firstly, 788 patients diagnosed with oral cancer voluntarily provided 5 ml of their peripheral blood and completed a self-administered questionnaire. Then, a total of 956 non-cancer healthy individuals as controls were selected by matching for age and gender after initial random sampling from the Health Examination Cohort of the hospital, and they also contributed their blood and completed the questionnaire. The questionnaire administered to each participant included questions on medical history and their individual frequency of alcohol consumption, areca chewing and smoking habit. Self-reported alcohol consumption, areca chewing and smoking habits were evaluated and classified as categorical variables. Information on these factors obtained as more than twice a week for years was recorded as "ever''. The male *versus* female ratio was 76% to 24% in each group, perfectly matched with each other. The recurrence and metastasis status of each patient were closely followed at least twice per year after their surgery. The mean age of the patients and the controls was 55.8 (SD=9.9) and 56.6 (SD=8.7) years, showing that the matching was successful, causing a non-significantly differential distribution between the case and control groups. More detailed information is summarized in Table I.

Genotyping processes. The genomic DNA from the peripheral blood leukocytes donated by each participant was prepared within 24 h after collection applying the QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, ROC), and stored at −80˚C until processed as per our previous articles (3-5). In this study, the genotypes at -1306 and -735 polymorphic sites in the *MMP2* promoter region were determined for all the individuals in both the control and oral cancer groups. In brief, the polymorphic sites were genotyped by typical polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodologies using a BioRad Mycycler (BioRad, Hercules, CA, USA). Each PCR reaction consisted of an initial cycle at 94˚C for 5 min; 40 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s; and a final extension at 72˚C for 10 min. After PCR, the SNP-containing DNA amplicons were subjected to individual overnight digestion by restriction endonucleases. Following digestion, each sample was immediately analyzed by agarose gel electrophoresis. All the genotypic processing was repeated by two researchers independently, and blindly, and the results were 100% concordant. The details of primer sequences and the restriction enzymes are provided in Table II.

Statistical analysis. The Student's *t*-test was used for comparing the distribution of ages between the two groups. Pearson's chi-square test was applied to compare the distribution of the *MMP2* -1306 and -735 genotypes among the subgroups, and also to examine the possible interaction among the indices of interest. The associations between the *MMP2* -1306 and -735 genotypes and oral cancer risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from logistic regression analysis. Any difference with an outcome of $p<0.05$ was considered statistically significant.

Results

The frequency distributions of selected demographic characteristics including age, gender, personal habits and primary tumor sites for the 788 patients with oral cancer and 956 non-cancer controls are summarized in Table I. Since we applied frequency matching for age and gender to recruit the non-cancer healthy controls, there was no difference in the distributions of age and gender between the control and case

Table II. *Summary of the primers, restriction enzymes and amplicon size after enzyme cutting for matrix metalloproteinase-2 (MMP2) genotyping polymerase chain reaction-restriction fragment length polymorphism conditions.*

Polymorphic site	Primer sequences	Restriction enzyme	Amplicon size after cutting, bp
MMP2 -1306	Forward 5'-CTTCCTAGGCTGGTCCTTACTGA-3'	*Xsp*I	C: 188+5
	Reverse 5'-CTGAGACCTGAAGAGCTAAAGAGCT-3'		T: 162+26+5
MMP2 -735	Forward 5'-GGATTCTTGGCTTGGCGCAGGA-3'	*Hinf*I	C: 391
	Reverse 5'-GGGGGCTGGGTAAAATGAGGCTG-3'		T: 338+53

groups (Table I). For these investigated individuals, betel quid chewers and smokers were found at higher percentages in patients with oral cancer than in the controls (Table I). The most frequently diagnosed primary tumors occurred in the tongue (41.2%) and buccal mucosa (37.3%) for patients with oral cancer in Taiwan.

The distributions of the *MMP2* promoter 1306 and -735 genotypes among the non-cancer controls and the patients with oral cancer are presented and statistically analyzed in Table II.

The genotypes of *MMP2* promoter 1306 were differently distributed between oral cancer and non-cancer control groups (p for trend=4.3×10^{-6}) (Table III, top). In detail, the *MMP2* promoter 1306 heterozygous CT and homozygous TT were both associated with reduced oral cancer risk (p=0.0001 and 0.0192, respectively; Table III). On the contrary, the genotypes of *MMP2* promoter 735 were not differently distributed between oral cancer and non-cancer control groups (p for trend=0.8932) (Table III).

To confirm the findings in Table III, the analysis of allelic frequency distribution for *MMP2* promoter 1306 and -735 was also conducted and the results are summarized in Table IV. Supporting the findings that heterozygous CT and homozygous TT genotypes of *MMP2* promoter 1306 were associated with oral cancer risk, the variant T allele was found at 10.9% in the case group, significantly lower than that of 16.7% in the control group ($p=1.1\times10^{-6}$). To sum up, there was a significant difference in the allelic frequencies of *MMP2* promoter 1306 between the control and oral cancer groups (Table IV). It was also validated that there was no significant differential distribution (p=0.6604) for the allelic frequencies of *MMP2* promoter -735 (Table IV).

Next, we were interested whether the *MMP2* promoter 1306 and -735 genotypes could serve as a predictor for the prognosis of patients with oral cancer. To fulfill this, the distributions of the *MMP2* promoter 1306 and -735 genotypes were examined among the patients stratified by disease recurrence and metastasis status with a cut-off of 5 years. Firstly, the patients with oral cancer carrying the genotype of CT or TT at *MMP2* promoter 1306 were at lower risk of metastasis within 5 years of surgery (p=0.0102) than those patients carrying the wild-type CC genotype at *MMP2* promoter 1306 (Table V). On the contrary, there was no differential distribution of the *MMP2* promoter 1306 genotype between patients with and those without recurrence within 5 years (Table V). There was no positive evidence for the involvement of *MMP2* promoter 735 genotype in determining the recurrence or metastasis status for these Taiwanese patients with oral cancer (data not shown).

Discussion

In the current case–control association study, the contribution of *MMP2* promoter 1306 and -735 genotypes to oral cancer risk was firstly evaluated among Taiwanese, where the male oral cancer density is highest in the world. The two SNP loci, -1306 and -735, are both located upstream of the *MMP2* transcriptional start site. Their variation might destroy the binding site of SP1, resulting in reduction of gene transcription, and eventually reduce the expression of MMP2 (20). The results showed that both the genotypic and the allelic frequencies of *MMP2* promoter 1306 were differentially distributed between the 788 patients with oral cancer and 956 non-cancer healthy controls (Tables III and IV). In addition, the variant T-bearing genotypes at *MMP2* promoter 1306 were associated with reduced risk of cancer metastasis in addition to cancer susceptibility itself (Table V).

MMP2 protein, also called gelatinase, is involved in the degradation of the intact fibrillar collagen, elastin, endothelin, fibroblast growth factor, MMP9, MMP13, plasminogen, and transforming growth factor β (21). MMP2 has been reported to play an important role in ECM degradation, which is important for primary tumor cells to undergo invasion and migration (22, 23). Mounting evidence indicates that activated MMP2 is observed and linked with poor prognosis of many types of cancer including melanoma, colorectal, breast, ovarian, lung and prostate cancer, reviewed and summarized in (24). MMP2 is thought to promote epithelial–mesenchymal transition through the degradation of type IV collagen, the most abundant component of the basement membrane. The basement membrane is important for maintaining tissue organization

Table III. *Distribution of matrix metalloproteinase-2 (MMP2) genotypes among the patients with oral cancer and non-cancer controls.*

	Controls		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
MMP2 -1306						
CC	657	68.7%	623	79.0%	1.00 (Reference)	
CT	279	29.2%	158	20.1%	0.60 (0.48-0.75)	**0.0001**
TT	20	2.1%	7	0.9%	0.37 (0.16-0.88)	**0.0192**
P_{trend}						**4.3×10^{-6}**
MMP2 -735						
CC	632	66.1%	515	65.4%	1.00 (Reference)	
CT	282	29.5%	235	29.8%	1.02 (0.83-1.26)	0.8333
TT	42	4.4%	38	4.8%	1.11 (0.71-1.75)	0.6513
P_{trend}						0.8932

[a]Based on Chi-square test without Yates' correction. Statistically significant values are shown in bold.

Table IV. *Distribution of allelic frequencies for matrix metalloproteinase-2 (MMP2) among patients with oral cancer and non-cancer controls.*

	Controls		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
MMP2 -1306						
C	1,593	83.3%	1,404	89.1%	1.00 (Reference)	
T	319	16.7%	172	10.9%	0.61 (0.50-0.75)	**1.1×10^{-6}**
MMP2 -735						
C	1,546	80.9%	1,265	80.3%	1.00 (Reference)	
T	366	19.1%	311	19.7%	1.04 (0.88-1.23)	0.6604

[a]Based on chi-square test without Yates' correction. Statistically significant values are shown in bold.

Table V. *Association of matrix metalloproteinase-2 (MMP2) genotypes with cancer recurrence and metastasis status.*

Patient status	*MMP2* C-1306T genotype			
	CC	CT+TT	OR (95% CI)	*p*-Value[a]
Recurrence status				
No recurrence >5 years	573	147	1.00 (Reference)	
Recurrence ≤5 years	50	18	1.40 (0.79-2.48)	0.2409
Metastasis status				
No metastasis >5 years	561	159	1.00 (Reference)	
Metastasis ≤5 years	62	6	0.34 (0.15-0.80)	0.0102

[a]Based on Chi-square test without Yates' correction. Statistically significant values are shown in bold.

and providing structural support for cells in addition to influencing cell signaling and polarity. It is also reported that basement membrane breakage is an essential step for the initiation of invasive and metastatic behaviors of most types of cancer (25). In a hamster model of tongue cancer, MMP1 and TIMP1, together with MMP2 and TIMP2, were shown to gradually increase with progression of tongue cancer (26). The invasive and metastatic capacity of oral cancer cells and lymph node metastasis in mouse oral cancer models were all closely related to the expression levels of MMP2 and MMP9

(27-29). In literature, there are only two articles investigating the contribution of MMP2 genotypes to oral cancer. In 2004, Lin and colleagues reported that the frequency of the CC genotype at *MMP2* -1306 was significantly higher in oral squamous cell carcinoma cases than in controls (p=0.04) (30). In 2006, O-Charoenrat and colleagues further assessed the expression level of MMP2 in serum association in addition to the contribution of *MMP2* -1306 genotypes to the risk of head and neck cancer (31). They found that the C and T allelic frequencies were 93.1% and 6.9%, respectively, in patients, compared with 87.2% and 12.8%, respectively, in controls, and the CC genotype frequency was significantly higher in patients than in controls (86.2% *vs*. 76%, p<0.05). Moreover, they found that the expression level of MMP2 in head and neck cancer cells carrying the CC genotype was significantly higher than that in cells carrying the CT genotype. Our findings are consistent with their mentioning that the T-allele of *MMP2* -1306 may serve as a protective marker. However, because of their moderate sample numbers (controls:cases=288/242 and 496/478, respectively) and limited number of studies, this conclusion should be interpreted with caution and further studies, especially those with larger samples, are needed to validate all the findings above.

ECM and MMPs are all important to the etiology of oral cancer. Previously, we investigated the contribution of genomic variants of other MMPs to oral cancer susceptibility among Taiwanese. In 2016, we found that 1G/2G genotype of *MMP1* promoter -1607 had a protective effect on oral cancer risk for smokers (9). Subsequently, we showed that genotypes at *MMP8* C-799T, Val436Ala (32) and MMP7 C-153T (33) appear not to play a major role in mediating personal risk of oral cancer. The contribution of the genotypes of other MMPs, especially for those whose proteins were proved to be differentially expressed in tumoral and non-tumoral sites, should be examined. In addition, the status of each MMP are also under the control of a complex network at several levels, including through their interactions with specific inhibitors, *e.g*. the tissue inhibitors of metalloproteinases (TIMPs) (11). Taking MMP2 as an example, the dynamic balance between MMP2 and TIMP2 plays a pivotal role in the maintenance of normal physiological conditions for cells, but it seems that the balance between MMP2 and TIMP2 in oral tissues is not as simple as a 'see-saw' relationship. In the near future, an overall analysis of *MMP2* and *TIMP2* genotype/phenotype may provide further evidence for evaluating the contribution of these genotypes to oral carcinogenesis.

In conclusion, these results provide evidence showing that the variant CT and TT genotypes at *MMP2* promoter 1306 were protective biomarkers for determining not only susceptibility to oral cancer, but also metastatic behavior in prognosis for Taiwanese.

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