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Polymorphisms and Plasma Levels of Tissue Inhibitor of Metalloproteinase-3: Impact on Genetic Susceptibility and Clinical Outcome of Oral Cancer

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Abstract: Oral cancer, the fourth most common cancer among men in Taiwan, is associated with environmental carcinogens. Tissue inhibitor of metalloproteinase-3 (TIMP3), a member of the TIMP family, is the only protein that binds to the extracellular matrix for suppressing cancer cell growth, angiogenesis, migration, and invasion. The association of TIMP3 polymorphism with oral cancer susceptibility, however, has not yet been reported. In this study, 1947 participants—1200 healthy male controls and 747 male patients with oral cancer—were recruited. Allelic discrimination of TIMP3 −1296 T > C (rs9619311), TIMP3 C > T (rs9862), and TIMP3 C > T (rs11547635) polymorphisms were assessed through real-time polymerase chain reaction. The authors discovered that individuals carrying the polymorphic rs9862 allele are more susceptible to oral cancer [odds ratio (OR), 1.5; 95% confidence interval (CI), 1.2-1.9; adjusted OR (AOR), 1.6; 95% CI, 1.2−2.1] after adjustment for betel quid chewing, alcohol, and tobacco consumption. Among 601 betel quid chewers, the TIMP3 polymorphism rs9862 T/T carriers had a 32.2-fold (95% CI, 20.2−51.3) increased oral cancer risk compared with those carrying C/C and not chewing betel quid. In addition, the authors observed a significant association between rs9862 variants and large tumors (OR, 1.5; 95% CI, 1.0−2.3) development. Moreover, TIMP3 plasma levels significantly increased in oral cancer patients who have large tumor or carry T allele rs9862 polymorphism. In conclusion, these results suggest that gene-environment interactions between the TIMP3 rs9862 polymorphisms and betel quid may alter oral cancer susceptibility and tumor growth in Taiwanese men.

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Abbreviations: ELISA = enzyme-linked immunosorbent assay, OSCC = oral squamous cell carcinoma, SNP = single nucleotide polymorphism, TIMP3 = tissue inhibitor of metalloproteinase-3.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy, the fourth most common cancer among men, and the sixth leading cause of cancer deaths in Taiwan.[1] Failure to control the primary cancer and lymph node metastasis are the main causes of death among patients with OSCC.[2] Development of OSCC is a multistep process mediated by both environmental risk factors and genetic factors. Betel quid chewing, tobacco use, and alcohol consumption are 3 common OSCC environmental risk factors. The combination of these environmental risk factors and certain gene polymorphisms may increase oral cancer susceptibility.[3] Gene expression is affected by single nucleotide polymorphism (SNP), which is a variation in the DNA sequence that occurs when a nucleotide (A, T, C, or G) changes more than 1% within a population. Previous studies have reported that SNPs located within a promoter or other regulatory regions of genes are associated with the development of certain diseases,[4] and several SNPs have been reported as predictive factors for a high OSCC risk.[5]

Tissue inhibitor of metalloproteinase-3 (TIMP3) is a member of the TIMP family; it is a 24 kDa secretory protein, and unlike its other family members, it binds firmly to the extracellular matrix. In addition, TIMP3 has a broad metalloproteinase inhibitory activity against matrix metalloproteinase members, a disintegrin and metalloproteinases (ADAM), and ADAM with thrombospondin domain (ADAM-TS) families.[6,7] A previous study showed that in head and neck squamous cell carcinomas (HNSCCs), TIMP3 mRNA expression was considerably higher in the HNSCC-associated stroma than in the stroma adjacent to the dysplastic or normal epithelia, and these high levels considerably reduced the overall survival rate.[8] Tissue inhibitor of metalloproteinase-3 hypermethylation has been reported in HNSCC and is related to the risk of developing second primary carcinomas.[9] Moreover, other report indicated that TIMP3 was hypermethylated in approximately 90% of clinically T1 and T2 OSCC cases.[10]

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Tissue inhibitor of metalloproteinase-3 is separately located on chromosome 22q12.1. Polymorphic variations in the TIMP3 exon region were associated with the survival rate of patients with adenocarcinoma.[11] Nevertheless, no studies have focused on the association between TIMP3 polymorphisms and solid tumor development. The TIMP3C allele promoter polymorphism at −1296 T > C (rs9619311) has been reported in patients with breast cancer and hepatocellular carcinoma (HCC),[12,13] and 2 polymorphisms in the exon regions, including the C allele at 249 T > C (rs9862) and T allele at 261 C > T (rs11547635), were identified in patients with adenocarcinoma and intracranial aneurysm, respectively.[11,14] The roles of these 3 gene polymorphisms in the susceptibility of oral cancer, however, have not been investigated. In the current study, a case-control association study was performed for the aforementioned 3 SNPs located in the TIMP3 promoter or exon regions (Table 1) to analyze the role of TIMP3 polymorphisms in oral cancer susceptibility and pathologic development. To our knowledge, this is the first study that demonstrates a considerable association between TIMP3 polymorphisms and oral carcinogenesis in Taiwanese men.

MATERIALS AND METHODS

Patient Specimens

In 2007 to 2014, for the case group, we collected 747 male patients at Chung Shan Medical University Hospital and Changhua Christian Hospital in Taiwan. We chose 1200 noncancer individuals from Taiwan Biobank as the control group. For oral cancer group, medical information of the patients, including TNM clinical staging and histologic grade, was obtained from their medical records. Whole blood specimens collected from oral cancer patients were placed in tubes containing EDTA, which were immediately centrifuged and stored at −20 °C for further analyses.

Selection of Tissue Inhibitor of Metalloproteinase-3 Polymorphisms

In this study, the selection of 3 well-characterized common polymorphisms from TIMP3 gene is based on their wide associations with the development of cancer.[11–13] We included −1295T > C (rs9619311) in the promoter region. Rs9862 and rs11547635, which are located in the exon of TIMP3, were selected in this study because these 2 SNPs were found to modify the binding affinities.[11]

Real-Time Polymerase Chain Reaction for Genotyping

Tissue inhibitor of metalloproteinase-3 rs9619311 (assay IDs: C_1840822_10), rs9862 (assay IDs: C_3294861_10), and rs11547635 (assay IDs: C_3294860_10) polymorphisms were assessed using an ABI StepOnePlus ™ Real-Time PCR System and analyzed using SDS v3.0 software (Applied Biosystems, Foster City, CA) as previously described.[13]

Quantitative Analysis of Plasma Tissue Inhibitor of Metalloproteinase-3 Level

The TIMP3 expressions in the plasma samples were analyzed by TIMP3 Human enzyme-linked immunosorbent assay (ELISA) Kit (Abcam). Briefly, 100 μL of prepared standards and diluted samples were added to appropriate wells of ELISA plate. The further procedures were performed according to the manufacturer's instructions. The value of OD 450 nm was measured with a microtest plate spectrophotometer, and TIMP3 levels were quantified based on the standard curve constructed using recombinant human TIMP3.

Statistical Analysis

All analyses were conducted with SAS statistical software (Version 9.1, 2005; SAS Institute, Cary, NC). The AOR and 95% CIs of the association between the genotype frequencies and oral cancer risk were estimated using multiple logistic regression models after controlling for other covariates, such as age, alcohol consumption, tobacco consumption and betel quid chewing. Fisher exact test was used to compare the demographic characteristic distributions between the controls and patients with case group. $P < 0.05$ was considered statistically significant.

RESULTS

The statistical analysis of the demographic characteristics is shown in Table 2. We discovered that betel quid chewing ($P < 0.001$), cigarette smoking ($P < 0.001$), and tobacco use ($P < 0.001$) differed significantly between the controls and oral cancer patients. The genotype distributions and associations between oral cancer and TIMP3 polymorphisms are presented in Table 3. The highest distribution frequencies of TIMP3 rs9619311, rs9862, and rs11547635 alleles in men from both groups, oral cancer patients, and healthy controls recruited for this study, were homozygous for T/T, heterozygous for C/T, and

TABLE 1. Variants, Position, Function, Amino Acid and Changes of Observed Tissue Inhibitor of Metalloproteinase-3 Sequence Variations

Variable	Exon (Contiguous Position)		
Chromosome	**22:32800707**	**22:32857293**	**22:32857305**
cDNA position and nucleotide change	c.−1295T > C	c.249T > C	c.261C > T
mRNA position	—	1435	1447
dbSNPrs no.	rs9619311	rs9862	rs11547635
Function	Promoter	Synonymous	Synonymous
dbSNP allele	C/T	CAT > CAC	TCC > TCT
Protein residue	—	H [His] > H [His]	S [Ser] > S [Ser]
Codon position	—	3	3

SNP = single nucleotide polymorphism.

TABLE 2. The Distributions of Demographical Characteristics in 1200 Controls and 747 Patients With Oral Cancer

Variable	Controls (N = 1200)	Patients (N = 747)	*P* Value
Age (yrs)	Mean ± SD	Mean ± SD	$P = 0.097$
	53.91 ± 10.02	54.71 ± 11.14	
Betel quid chewing			
No	1001 (83.4%)	146 (19.5%)	$P < 0.001^*$
Yes	199 (16.6%)	601 (80.5%)	
Cigarette smoking			
No	564 (47.0%)	86 (11.5%)	$P < 0.001^*$
Yes	636 (53.0%)	661 (88.5%)	
Alcohol drinking			
No	963 (80.3%)	322 (43.1%)	$P < 0.001^*$
Yes	237 (19.8%)	425 (56.9%)	

Mann-Whitney *U* test or Fisher exact test was used between healthy controls and patients with oral cancer.
* *P* value < 0.05 as statistically significant.

homozygous for C/C, respectively. After adjusting for several variables, no significant differences were observed in oral cancer development in participants with TIMP3 rs9619311 and rs11547635 polymorphisms compared with that in wild type (WT) participants. Participants with the TIMP3 rs9862 T/T genotype, however, exhibited significantly ($P < 0.05$) higher OSCC development risks 1.618 (95% CI, 1.15–2.3) compared with the participants with TIMP3 rs9862 C/C (Table 3).

After revealing a significant association between the TIMP3 249T > C (rs9862) polymorphism and oral cancer susceptibly, we analyzed the combined effects of environmental factors and TIMP3 249T > C (rs9862) polymorphism on the oral cancer risk. In Table 4, among the 601 betel quid chewers in our study, the TIMP3 rs9862 TT homozygote was modified by exposure to betel quid chewing with an additive effect, suggesting that the TIMP3 249T > C polymorphism is associated with betel quid consumption and oral cancer susceptibility.

To clarify the effects of TIMP3 249T > C polymorphism on the oral cancer clinical status, such as clinical stage, primary tumor size, lymph node metastasis, and histologic grade, the distribution frequency of the clinical status and TIMP3 genotype frequencies in oral cancer patients were estimated. In this study, we classified the oral cancer patients consuming betel nut into 2 subgroups. In the first subgroup, patients had at least 1 C allele (C/C or C/T); in the other subgroup, patients had homozygous alleles T/T. In Table 5, patients with a homozygous T/T allele showed an increased risk of developing tumor size >T2 (OR = 1.5; 95% CI, 1.0–2.2). The rs9862 polymorphism, however, failed to show an association with the clinical stage, lymph node metastasis, and tumor differentiation, suggesting that rs9862 variants may affect the tumor cell proliferation but not invasion and differentiation.

To realize correlation between the plasma level of TIMP3 and rs9862 polymorphism, we used ELISA assays to analyze plasma TIMP3 levels in 262 OSCC patients. First, we analyze the plasma levels of betel quid consumption and showed that the mean plasma levels of TIMP3 increased in betel quid chewers (3432.7 ± 208.2 pg/mL) compare with those who did not practice betel quid chewing (2782.6 ± 435.3 pg/mL; Figure 1A). Among 216 betel quid chewers, plasma levels of TIMP3 was significantly associated with large tumor in OSCC patients ($P < 0.01$), the mean plasma level of TIMP3 is 2842.87 ± 259.58 pg/mL in small tumor (≦T2) and 3960.37 ± 311.73 pg/mL in large tumor (>T2; Figure 1B). Moreover, OSCC patient who carry C/T (3331.7 ± 282.2 pg/mL) and T/T (4924.4 ± 468.8 pg/mL) rs9862 polymorphism have significantly highly plasma levels of TIMP3 compare to C/C (2035.9 ± 266.7 pg/mL) genotype (Fig. 1C).

TABLE 3. Adjusted Odds Ratio and 95% Confidence Interval of Oral Cancer Associated With Tissue Inhibitor of Metalloproteinase-3 Genotypic Frequencies

Variable	Controls (N = 1200) n (%)	Patients (N = 747) n (%)	OR (95% CI)	AOR (95% CI)
rs9619311				
TT	995 (82.9%)	625 (83.7%)	1.00	1.00
TC	189 (15.8%)	115 (15.4%)	0.969 (0.753–1.247)	1.213 (0.867–1.695)
CC	16 (1.3%)	7 (0.9%)	0.697 (0.285–1.703)	1.047 (0.324–3.387)
TC + CC	205 (17.1%)	122 (16.3%)	0.947 (0.741–1.211)	1.201 (0.867–1.663)
rs9862				
CC	414 (34.5%)	192 (25.7%)	1.00	1.00
CT	556 (46.3%)	391 (52.3%)	1.516 (1.224–1.879)*	1.567 (1.182–2.076)*
TT	230 (19.2%)	164 (22.0%)	1.538 (1.181–2.001)*	1.618 (1.145–2.287)*
CT + TT	786 (65.5%)	555 (74.3%)	1.523 (1.243–1.865)*	1.582 (1.212–2.064)*
rs11547635				
CC	559 (46.6%)	367 (49.1%)	1.00	1.00
CT	523 (43.6%)	324 (43.4%)	0.944 (0.779–1.142)	0.893 (0.695–1.148)
TT	118 (9.8%)	56 (7.5%)	0.723 (0.512–1.020)	0.758 (0.484–1.189)
CT + TT	641 (53.4%)	380 (50.9%)	0.903 (0.752–1.084)	0.870 (0.684–1.106)

The OR with their 95% confidence intervals were estimated by logistic regression models. The adjusted AOR with their 95% confidence intervals were estimated by multiple logistic regression models after controlling for betel nut chewing, alcohol, and tobacco consumption. AOR = adjusted odds ratio, CI = confidence interval, OR = odds ratio.
* *p* value < 0.05 as statistically significant.

TABLE 4. Combined Effect of With Tissue Inhibitor of Metalloproteinase-3 rs9862 Genotypic Frequencies and Betel Chewing in Oral Cancer Risk

Variable	Controls (N = 1200) n (%)	Patients (N = 747) n (%)	OR (95% CI)	*P* value
rs9862/betel quid chewing				
CC/no	444 (29.6%)	50 (6.5%)	1.00	
CT/no	613 (40.9%)	85 (11.0%)	1.231 (0.850–1.783)	0.270
TT/no	244 (16.2%)	36 (4.6%)	1.310 (0.830–2.067)	0.245
CC/yes	69 (4.6%)	156 (20.1%)	20.077 (16.362–30.166)	<0.001
CT/yes	93 (6.2%)	314 (40.5%)	29.982 (20.651–43.530)	<0.001
TT/yes	37 (2.5%)	134 (17.3%)	32.160 (20.163–51.295)	<0.001

The OR with their 95% confidence intervals were estimated by logistic regression models. CI = confidence interval, OR = odds ratio.

DISCUSSION

Alcohol consumption, tobacco smoking, and betel quid chewing are the main known environmental risk factors of oral cancer.[15] In this study, the oral cancer group had a higher percentage of participants who were betel quid chewers and tobacco and alcohol consumers (80.5%, 88.5%, and 56.9%, respectively) than did the control group (16.6%, 53.0%, and 19.8%, respectively), indicating that betel quid chewing and tobacco and alcohol consumption are substantially associated with increased oral cancer risks. In the previous studies, Ko et al,[16] found that betel quid consumption contribute to oral cancer in Taiwan. In addition, lime-piper betel quid may increase protein levels of proto-oncogenes and indicate that it could be a tumor promoter.[17] Furthermore, in an animal model, hamsters fed with betel quid or areca nut slowed hyperkeratosis and acanthosis of cheek pouches.[18] This evidence suggests that environmental carcinogen exposure is involved with the onset and pathogenesis of oral cancer.

Tissue inhibitor of metalloproteinase-3 acts as a tumor suppressor gene in many cancers by inhibiting tumor growth, angiogenesis, invasion, and metastasis.[19–22] Moreover, a TIMP3 expression loss correlates with poor prognosis and survival in cancer patients.[23,24] A gene expression loss can be caused by different mechanisms, including genetic or epigenetic alternations. In epigenetic alternations, TIMP3 hypermethylation has been reported in patients with esophageal, gastric, kidney, and brain cancer.[23,25,26] Downregulation of TIMP3 in tumors can also be regulated by microRNAs, such as miR21, miR181b, miR221, and miR222.[27–29] Single nucleotide polymorphisms are genetic alternations, and TIMP3 polymorphisms have been reported to be associated with breast cancer, adenocarcinoma, and HCC.[11,13,30] Our study, however, is the first to report an association between TIMP3 polymorphisms and OSCC. The data in Table 3 shows that the men with TIMP3 polymorphism rs9862 T/T has higher risks for OSCC than do men with the C/C genotype.

In Taiwan, unlike the majority of global betel quid chewers, male adults chew betel quid without adding tobacco.[31–33] Several case-control studies have indicated that exposure to betel quid may partially be involved with the onset and pathogenesis of oral cancer.[34–36] Increasing evidence, however, demonstrates that genomic changes may more considerably lead cells to progress from the preneoplastic stage to cancer.[37] In addition, many studies have reported that the combination of gene polymorphism and betel quid chewing slightly increased the OSCC risks.[1,38,39] Betel quid chewers with a CYP26B1 polymorphism AA showed an increased

TABLE 5. Clinical Statuses and Tissue Inhibitor of Metalloproteinase-3 rs9862 Genotype Frequencies in Oral Cancer Among 601 Betel Quid Chewers

	TIMP3 rs9862 (betel quid chewers)			
Variable	CC + CT (N = 468) n (%)	TT (N = 133) n (%)	OR (95% CI)	*P* Value
Clinical Stage				
Stage I/II	229 (48.9%)	60 (45.1%)	1.00	*P* = 0.437
Stage III/IV	239 (51.1%)	73 (54.9%)	1.166 (0.792–1.716)	
Tumor size				
<T2	272 (58.1%)	63 (47.4%)	1.00	*P* = 0.028
>T2	196 (41.9%)	70 (52.6%)	1.542 (1.047–2.270)	
Lymph node metastasis				
No	306 (64.4%)	98 (73.7%)	1.00	*P* = 0.072
Yes	162 (34.6%)	35 (26.3%)	0.675 (0.439–1.037)	
Cell differentiation				
Well	78 (16.7%)	19 (14.3%)	1.00	*P* = 0.510
Moderate/poor	390 (83.3%)	114 (85.7%)	1.200 (0.697–2.066)	

CI = confidence interval, OR = odds ratio, TIMP3 = tissue inhibitor of metalloproteinase-3.

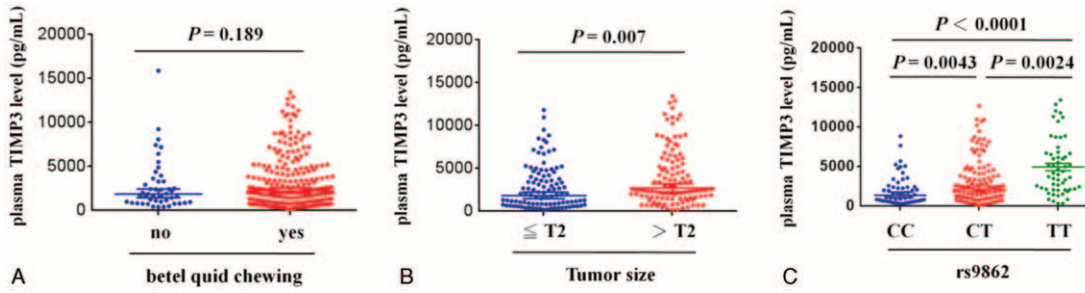

FIGURE 1. Enzyme linked immunosorbent assay-determined plasma TIMP3 level of OSCC patients. A, TIMP3 levels were compared according to betel quid consumption and results showed that TIMP3 levels were increased in betel nut chewers when compared with those without betel quid chewing. B, Among betel quid chewers, TIMP3 levels were compared according to tumor size and results showed that TIMP3 levels were significantly higher in large tumor (>T2) when compared with small tumor (≦T2). C, Among betel quid chewers, TIMP3 levels were compared according to rs9862 polymorphism and results showed that TIMP3 levels were significantly higher in patients who carry CT or TT genotypes when compared with patients who carry CC genotype. TIMP3 = Tissue inhibitor of metalloproteinase-3.

OSCC risk (AOR = 70.04; 95% CI, 13.6–360.1) compared with WT individuals who did not practice betel quid chewing.[39] Our previous study also indicated that reversion-inducing-cysteine-rich protein with kazal motifs (RECK) polymorphisms carriers with betel quid chewing habits have a 7.6-fold to 25.3-fold higher oral cancer risk compared with RECK WT carriers without betel quid chewing habits.[1] This study showed that the combined effect of TIMP3 rs9862T/T genotype and betel quid consumption significantly elevated the OSCC risk.

Single nucleotide polymorphism rs9619311 −1296 T > C is located in the TIMP3 promoter region. In our previous study, we revealed that TIMP3 rs9619311 genetic variants were significantly associated with the HCC susceptibility among women but not in men.[13] Also, Lei et al[12] analyzed the association between TIMP3 rs9619311 and breast cancer susceptibility and demonstrated that the C allele carriers had slightly increased levels of breast cancer susceptibility (OR = 1.25, 95% CI, 1.05–1.5). No association between the TIMP3 rs9619311 and breast cancer risk and patient survival has also been reported;[30] moreover, the TIMP3 rs9619311 distribution between patients with bladder cancer and healthy controls was not significantly different.[40] In this study, the TIMP3 rs9619311 did not show a substantial association with OSCC in Taiwanese men.

A tumor growth involves several major steps, including angiogenesis. Previous studies have demonstrated that angiogenesis is crucial in tumor progression, where the angiogenic activities are frequently correlated with tumor growth, metastasis, and the prognosis of patients with malignant neoplasms.[41,42] Tissue inhibitor of metalloproteinase-3 has several anticancer properties, such as the antiangiogenesis effect, where TIMP3 blocks the vascular endothelial growth factor binding to vascular endothelial growth factor receptor-2,[43] and restoration of TIMP3 in colorectal cancer cells has been reported to suppress the tumor growth.[44] Moreover, TIMP3 expression correlates with inhibition of directionally persistent endothelial cell migration and adversely affects the angiogenic potential and growth in melanomas.[21] In our study, TIMP3 rs9862 was considerably associated with large tumors. Although the data do not show the biologic mechanism of how TIMP3 affects tumor growth, rs9862 may have a functional role in influencing TIMP3 expression, activity, splicing, and epigenetic modification. Single nucleotide polymorphism rs9862 is present on the TIMP3 exon 3 without replacing the amino acids; however, a change in DNA sequence might affect binding ability of DNA-binding proteins. Bashash et al[11] used a gel shift assay to analyze the rs9862 function in adenocarcinoma patients, which suggested that SNP rs9862 influences an unidentified protein binding and may have a functional role in altering patient survival. Our ELISA data also demonstrated that plasma levels of TIMP3 was significantly increased in betel quid chewers of OSCC patient who carry a T allele rs9862 polymorphism or have large tumor. It is interesting that high plasma levels of TIMP3 contributed to poor outcomes for OSCC patients in our study. Similar results were also reported in Kornfeld et al,[8] they suggested that high TIMP3 mRNA levels were expressed in HNSCC-stroma than in the stroma adjacent to the dysplastic or normal epithelia, and high levels of TIMP3 showed significant reduction in the overall survival rate. Therefore, which protein binds to the aforementioned SNP region and the molecular mechanisms behind the SNP regulation of the tumor growth and TIMP3 expression in oral cancer warrant further investigation.

In conclusion, we systematically investigated 3 polymorphisms across TIMP3, and discovered the SNP rs9862 association with oral cancer susceptibility. In addition, a combined effect of SNP rs9862 and betel quid chewing contributes to the tumor growth. Although our study does not show rs9862 functional significance, these findings do support a possible TIMP3 role in oral cancer growth. Future studies that include the TIMP3 rs9862 polymorphism might contribute in predicting OSCC susceptibility and its pathologic development.

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