科技部補助專題研究計畫成果報告
期末報告

基質金屬蛋白酶組織抑制因子3在口腔癌致癌過程與表觀遺傳學的研究

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中 文 摘 要 ： 口腔癌為台灣男性四大常見的癌症之一，且與環境致癌物有密切相關性。基質金屬蛋白酶組織抑制劑3（TIMP3）為TIMP家族成員中唯一結合到細胞外基質的蛋白，具有用抑制癌細胞生長、血管生成、遷移和侵襲的功能，但有關 TIMP3 基因多型性（polymorphism）與口腔癌的相關性卻沒有被提到。因此，本篇研究收集了1947個樣本，包含1200健康的男性和747男性的口腔癌病人，利用即時聚合酶連鎖反應 （real-time PCR） 分析TIMP3 -1296 T> C （rs9619311）、TIMP3 C> T（rs9862）和TIMP3 C> T（rs11547635）三個位置的基因多型性。我們發現攜帶rs9862 TT基因型的人經校正後罹患口腔癌的風險值為正常人的1.6倍 (AOR=1.6; 95% CI, 1.2-2.1)，而在601位嚼檳榔者中，帶有rs9862 TT基因型的人相對於帶有CC基因型且沒有嚼檳榔者增加32.2倍罹患口腔癌的風險值 （OR=32.2; 95%CI, 20.2-51.3）。此外，我們觀察到rs9862 TT基因型與較大的腫瘤有顯著相關性（largest dimension > 2 cm; OR=1.5; 95%CI, 1.0-2.3）。在ELISA實驗中，我們分析了262個口腔癌患者，證實有嚼檳榔的患者其血漿中TIMP3蛋白的濃度比不嚼檳榔的高，而在216位嚼檳榔者中，血漿中TIMP3蛋白的濃度在較大腫瘤或攜帶rs9862 T基因型的口腔癌病人中有顯著的增加。因此，本篇研究認為，TIMP3 rs9862多型性與檳榔之間的相互作用可視為台灣男性口腔癌發生及癌細胞生長的可能因子

中 文 關 鍵 詞 ： 口腔癌,基因多型性,基質金屬蛋白酶組織抑制劑3

英 文 摘 要 ： Oral cancer, the fourth most common cancer among males 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. However, the association of TIMP3 polymorphism with oral cancer susceptibility 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 (PCR). We discovered that individuals carrying the polymorphic rs9862 allele are more susceptible to oral cancer (odds ratio [OR], 1.5; 95% 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, we 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.

英文關鍵詞：oral cancer, polymorphism, TIMP3

Polymorphisms and plasma levels of TIMP3: impact on genetic susceptibility and clinical outcome of oral cancer

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Running Title: TIMP3 Polymorphisms in the Risk of OSCC

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Abstract

Oral cancer, the fourth most common cancer among males 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. However, the association of *TIMP3* polymorphism with oral cancer susceptibility 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 (PCR). We discovered that individuals carrying the polymorphic rs9862 allele are more susceptible to oral cancer (odds ratio [OR], 1.5; 95% 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, we 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.

Keywords: Oral cancer; TIMP3; polymorphism

Abbreviations:

ELISA=enzyme-linked immunosorbent assay, OSCC = Oral squamous cell carcinoma, TIMP3=Tissue inhibitor of metalloproteinase-3, SNP=single nucleotide polymorphism

Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy, the fourth most common cancer among males, 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 (ECM). In addition, TIMP3 has a broad metalloproteinase inhibitory activity against matrix metalloproteinase (MMP) 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]. TIMP3 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].

TIMP3 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 *TIMP3* C 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]. However, the roles of these 3 gene polymorphisms in the susceptibility of oral cancer have not been investigated. In the present 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 pathological 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 2007–2014, for the case group, we recruited 747 male patients at Chung Shan Medical University Hospital in Taichung and Changhua Christian Hospital in Changhua, Taiwan. For the control group, we randomly chose 1200 non-cancer individuals from Taiwan Biobank. For both groups, we administered a questionnaire to obtain information on their exposure to betel quid chewing, tobacco use, and alcohol consumption. Medical information of the patients, including TNM clinical staging, primary tumor size, lymph node involvement, and histologic grade, was obtained from their medical records. Oral cancer patients were clinically staged at the time of their diagnoses according to the TNM staging system of the *American Joint Committee on Cancer (AJCC) Staging Manual* (*Seventh edition*), where Stage I = T1N0M0; Stage II = T2N0M0; Stage III = T3N0M0, or T1, T2, or T3N1M0; Stage IV = any T4 lesion, any N2 or N3 lesion, or any M1 lesion. Tumor differentiation was examined by a pathologist according to the AJCC classifications. Whole blood specimens of the controls and OSCC patients were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), which were immediately centrifuged and stored at −20℃. This study was approved by the Institutional Review Board of Chung-Shan Medical University Hospital, and informed written consent to participate

in the study was obtained from each participant.

Selection of TIMP3 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 since these 2 SNPs were found to modify the binding affinities [11].

Real-Time PCR

The allelic discrimination of *TIMP3* rs9619311, rs9862, and rs11547635 polymorphisms were assessed using an ABI StepOne TM Real-Time PCR System (Applied Biosystems, Foster City, CA) and analyzed using SDS v3.0 software (Applied Biosystems, Foster City, CA) by selecting the TaqMan assay (assay IDs: C_27084758_10). The final volume for each reaction was 5 μL, containing 2.5 μL TaqMan Genotyping Master Mix, 0.125 μL TaqMan probes mix, and 10 ng genomic DNA. The real-time PCR reaction included an initial denaturation step at 95℃ for 10 min, followed by 40 cycles, each consisting of a denaturation step at 95℃ for 15 s and an annealing step at 60℃ for 1 min.

Quantitative Analysis of Plasma TIMP3 Level

The TIMP3 levels in the plasma samples were analyzed by TIMP3 Human enzyme-linked immunosorbent assay (ELISA) Kit (Abcam). 100 μL of prepared standards and diluted samples was added to appropriate wells of ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microtest plate spectrophotometer, and TIMP3 levels were quantified with a calibration curve using human TIMP3 as a standard.

Statistical Analysis

Hardy–Weinberg equilibrium was calculated using a chi-square goodness-of-fit test for biallelic markers. Mann–Whitney U-test and Fisher's exact test were used to compare the age differences and demographic characteristic distributions between the controls and patients with oral cancer. The adjusted odds ratio and 95% CIs of the association between the genotype frequencies and oral cancer risk and the clinical pathological characteristics were estimated using multiple logistic regression models after controlling for other covariates, such as age (year), betel quid chewing (ever- versus never-user), alcohol (current heavy drinker versus current not heavy drinker), and tobacco consumption (smoker versus nonsmoker). $P < 0.05$ was considered significant. The data were analyzed on SAS statistical software (Version 9.1, 2005; SAS Institute, Cary, NC).

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 males from both groups, oral cancer patients and healthy controls recruited for this study, were homozygous for T/T, heterozygous for C/T, and 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. However, participants with the *TIMP3* rs9862 T/T genotype 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 one 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). However, the rs9862 polymorphism 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 to those who did not practice betel quid chewing (2782.6 ±

435.3 pg/mL) (Fig. 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) (Fig. 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).

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 previously studies, Ko et al., found that betel quid consumption contribute to oral cancer in Taiwan [16]. 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.

TIMP3 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]. SNPs are genetic alternations, and *TIMP3* polymorphisms have been reported to be associated with breast cancer, adenocarcinoma, and hepatocellular carcinoma [11, 13, 30]. However, our study is the first to report an association between *TIMP3* polymorphisms and OSCC. The data in Table 3 shows that the males with *TIMP3* polymorphism rs9862 T/T has higher risks for OSCC than do males 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]. However, increasing evidence demonstrates that genomic changes may more considerably lead cells to progress from the preneoplastic stage to cancer [37]. Additionally, 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 OSCC risk (AOR = 70.04; 95% CI, 13.6-360.1) compared with wild type individuals who did not practice betel quid chewing [39]. Our previous study also indicated that *RECK* polymorphisms carriers with betel quid chewing habits have a 7.6-fold (95% CI,

2.96-19.6) to 25.3-fold (95% CI, 9.6-67.02) higher oral cancer risk compared with RECK wild type carriers without betel quid chewing habits [1]. This study showed that the combined effect of *TIMP3* rs9862 T/T genotype and betel quid consumption significantly elevated the OSCC risk.

SNP rs9619311 −1296 T>C is located in the *TIMP3* promoter region. In our previous study, we revealed that *TIMP3* polymorphism −1296 T>C genetic variants were significantly associated with the HCC susceptibility among women but not in men [13]. Lei et al [12] recruited 959 patients with breast cancer and 952 healthy controls in Sweden to analyze the association between *TIMP3* −1296 T>C polymorphisms 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). However, Peterson et al [30] revealed no association between the *TIMP3* −1296 T>C polymorphism and breast cancer risk and patient survival; moreover, the *TIMP3* −1296 T>C polymorphism distribution between patients with bladder cancer and healthy controls was not significantly different [40]. In this study, the *TIMP3* polymorphism −1296 T>C did not show a substantial association with OSCC in Taiwanese men.

A tumor growth of involves several major steps, including angiogenesis. Previous studies have demonstrated that tumor-associated angiogenesis is crucial in

tumor progression, where the angiogenic activities are frequently correlated with tumor growth, lymph node metastasis, distant metastasis, and the prognosis of patients with malignant neoplasms [41, 42]. TIMP3 has several anticancer properties, such as the antiangiogenesis effect, where TIMP3 blocks the vascular endothelial growth factor (VEGF) binding to VEGF 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 biological mechanism of how TIMP3 affects tumor growth, rs9862 may have a functional role in influencing TIMP3 expression, activity, splicing, and epigenetic modification. SNP 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's 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 significantly reduced the overall survival rate. Therefore, further investigating 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 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 pathological development.

References:

1. Chung TT, Pan MS, Kuo CL, et al. Impact of RECK gene polymorphisms and environmental factors on oral cancer susceptibility and clinicopathologic characteristics in Taiwan. Carcinogenesis 2011;32(7):1063-8.
2. Scully C, Porter S. ABC of oral health. Oral cancer. BMJ (Clinical research ed) 2000;321(7253):97-100.
3. Weng CJ, Lin CW, Chung TT, Tsai CM, Chen MK, Yang SF. Impact of uPA system gene polymorphisms on the susceptibility of environmental factors to carcinogenesis and the development of clinicopathology of oral cancer. Annals of surgical oncology 2011;18(3):805-12.
4. Shastry BS. SNP alleles in human disease and evolution. Journal of human genetics 2002;47(11):561-6.
5. Brunotto M, Zarate AM, Bono A, Barra JL, Berra S. Risk genes in head and neck cancer: a systematic review and meta-analysis of last 5 years. Oral oncology 2014;50(3):178-88.
6. Woessner JF, Jr. That impish TIMP: the tissue inhibitor of metalloproteinases-3. The Journal of clinical investigation 2001;108(6):799-800.
7. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochimica et biophysica acta 2010;1803(1):55-71.
8. Kornfeld JW, Meder S, Wohlberg M, et al. Overexpression of TACE and TIMP3 mRNA in head and neck cancer: association with tumour development and progression. British journal of cancer 2011;104(1):138-45.
9. Rettori MM, de Carvalho AC, Longo AL, et al. TIMP3 and CCNA1 hypermethylation in HNSCC is associated with an increased incidence of second primary tumors. Journal of translational medicine 2013;11:316.
10. Arantes LM, de Carvalho AC, Melendez ME, et al. Validation of methylation markers for diagnosis of oral cavity cancer. European journal of cancer (Oxford, England : 1990) 2015;51(5):632-41.
11. Bashash M, Shah A, Hislop G, et al. Genetic polymorphisms at TIMP3 are associated with survival of adenocarcinoma of the gastroesophageal junction. PloS one 2013;8(3):e59157.
12. Lei H, Hemminki K, Altieri A, et al. Promoter polymorphisms in matrix metalloproteinases and their inhibitors: few associations with breast cancer susceptibility and progression. Breast cancer research and treatment 2007;103(1):61-9.
13. Tsai HT, Hsieh MJ, Chiou HL, et al. TIMP-3 -1296 T>C and TIMP-4 -55 T>C gene

polymorphisms play a role in the susceptibility of hepatocellular carcinoma among women. Tumour Biol 2014;35(9):8999-9007.
14. Krex D, Rohl H, Konig IR, Ziegler A, Schackert HK, Schackert G. Tissue inhibitor of metalloproteinases-1, -2, and -3 polymorphisms in a white population with intracranial aneurysms. Stroke; a journal of cerebral circulation 2003;34(12):2817-21.
15. Radoi L, Luce D. A review of risk factors for oral cavity cancer: the importance of a standardized case definition. Community dentistry and oral epidemiology 2013;41(2):97-109, e78-91.
16. Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM, Tsai CC. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 1995;24(10):450-3.
17. Lin MH, Wang CJ, Huang HP, Chou MY, Chou FP. The tumorigenic characteristics of lime-piper betel quid-transformed JB6 cells. Archives of toxicology 2004;78(3):167-73.
18. Chiang CP, Chang MC, Lee JJ, et al. Hamsters chewing betel quid or areca nut directly show a decrease in body weight and survival rates with concomitant epithelial hyperplasia of cheek pouch. Oral oncology 2004;40(7):720-7.
19. Anania MC, Sensi M, Radaelli E, et al. TIMP3 regulates migration, invasion and in vivo tumorigenicity of thyroid tumor cells. Oncogene 2011;30(27):3011-23.
20. Zhang L, Zhao L, Zhao D, et al. Inhibition of tumor growth and induction of apoptosis in prostate cancer cell lines by overexpression of tissue inhibitor of matrix metalloproteinase-3. Cancer gene therapy 2010;17(3):171-9.
21. Das AM, Seynhaeve AL, Rens JA, et al. Differential TIMP3 expression affects tumor progression and angiogenesis in melanomas through regulation of directionally persistent endothelial cell migration. Angiogenesis 2014;17(1):163-77.
22. Cruz-Munoz W, Sanchez OH, Di Grappa M, English JL, Hill RP, Khokha R. Enhanced metastatic dissemination to multiple organs by melanoma and lymphoma cells in timp-3-/- mice. Oncogene 2006;25(49):6489-96.
23. Darnton SJ, Hardie LJ, Muc RS, Wild CP, Casson AG. Tissue inhibitor of metalloproteinase-3 (TIMP-3) gene is methylated in the development of esophageal adenocarcinoma: loss of expression correlates with poor prognosis. International journal of cancer Journal international du cancer 2005;115(3):351-8.
24. Wu DW, Tsai LH, Chen PM, et al. Loss of TIMP-3 promotes tumor invasion via elevated IL-6 production and predicts poor survival and relapse in HPV-infected non-small cell lung cancer. The American journal of pathology 2012;181(5):1796-806.
25. Kang SH, Choi HH, Kim SG, et al. Transcriptional inactivation of the tissue inhibitor of metalloproteinase-3 gene by dna hypermethylation of the 5'-CpG island

in human gastric cancer cell lines. International journal of cancer Journal international du cancer 2000;86(5):632-5.
26. Bachman KE, Herman JG, Corn PG, et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer research 1999;59(4):798-802.
27. Gabriely G, Wurdinger T, Kesari S, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Molecular and cellular biology 2008;28(17):5369-80.
28. Garofalo M, Di Leva G, Romano G, et al. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer cell 2009;16(6):498-509.
29. Wang B, Hsu SH, Majumder S, et al. TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene 2010;29(12):1787-97.
30. Peterson NB, Beeghly-Fadiel A, Gao YT, et al. Polymorphisms in tissue inhibitors of metalloproteinases-2 and -3 and breast cancer susceptibility and survival. International journal of cancer Journal international du cancer 2009;125(4):844-50.
31. Mack TM. The new pan-Asian paan problem. Lancet (London, England) 2001;357(9269):1638-9.
32. Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer 2004;85:1-334.
33. Gupta PC, Warnakulasuriya S. Global epidemiology of areca nut usage. Addiction biology 2002;7(1):77-83.
34. Lu CT, Yen YY, Ho CS, et al. A case-control study of oral cancer in Changhua County, Taiwan. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 1996;25(5):245-8.
35. Merchant A, Husain SS, Hosain M, et al. Paan without tobacco: an independent risk factor for oral cancer. International journal of cancer Journal international du cancer 2000;86(1):128-31.
36. Chen PC, Kuo C, Pan CC, Chou MY. Risk of oral cancer associated with human papillomavirus infection, betel quid chewing, and cigarette smoking in Taiwan--an integrated molecular and epidemiological study of 58 cases. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 2002;31(6):317-22.
37. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nature genetics 2002;31(4):339-46.

38. Chen MK, Chiou HL, Su SC, et al. The association between hypoxia inducible factor-1alpha gene polymorphisms and increased susceptibility to oral cancer. Oral oncology 2009;45(12):e222-6.
39. Chen PH, Lee KW, Chen CH, et al. CYP26B1 is a novel candidate gene for betel quid-related oral squamous cell carcinoma. Oral oncology 2011;47(7):594-600.
40. Wieczorek E, Reszka E, Jablonowski Z, et al. Genetic polymorphisms in matrix metalloproteinases (MMPs) and tissue inhibitors of MPs (TIMPs), and bladder cancer susceptibility. BJU international 2013;112(8):1207-14.
41. Folkman J. Tumor angiogenesis: therapeutic implications. The New England journal of medicine 1971;285(21):1182-6.
42. Folkman J. Role of angiogenesis in tumor growth and metastasis. Seminars in oncology 2002;29(6 Suppl 16):15-8.
43. Qi JH, Ebrahem Q, Moore N, et al. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nature medicine 2003;9(4):407-15.
44. Lin H, Zhang Y, Wang H, et al. Tissue inhibitor of metalloproteinases-3 transfer suppresses malignant behaviors of colorectal cancer cells. Cancer gene therapy 2012;19(12):845-51.

Table 1. Variants, position, function, amino acid and changes of observed TIMP3 sequence variations.

Variable	Exon (contiguous position)		
Chromosome	22:32800707	22:32857293	22:32857305
cDNA position & nucleotide change	c.-1295T>C	c.249T>C	c.261C>T
mRNA position	----	1435	1447
dbSNP rs 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

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 ± S.D.	Mean ± S.D.	
	53.91 ± 10.02	54.71 ± 11.14	p=0.097
Betel quid chewing			
No	1001 (83.4%)	146 (19.5%)	
Yes	199 (16.6%)	601 (80.5%)	p <0.001*
Cigarette smoking			
No	564 (47.0%)	86 (11.5%)	
Yes	636 (53.0%)	661 (88.5%)	p <0.001*
Alcohol drinking			
No	963 (80.3%)	322 (43.1%)	
Yes	237 (19.8%)	425 (56.9%)	p <0.001*

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

Table 3. Adjusted odds ratio (AOR) and 95% confidence interval (CI) of oral cancer associated with *TIMP3* 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 odds ratio (OR) with their 95% confidence intervals were estimated by logistic regression models.

The adjusted odds ratio (AOR) with their 95% confidence intervals were estimated by multiple logistic regression models after controlling for betel nut chewing, alcohol and tobacco consumption.

Table 4. Combined effect of with *TIMP3* rs9862 genotypic frequencies and betel chewing in oral cancer risk.

Variable	Controls (n=1200)(%)	Patients (n=747) (%)	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 odds ratios (OR) with their 95% confidence intervals were estimated by logistic regression models.

Table 5. Clinical statuses and TIMP3 rs9862 genotype frequencies in oral cancer among 601 betel quid chewers.

Variable	**TIMP3 rs9862 (betel quid chewers)**			
	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)	

Figure 1

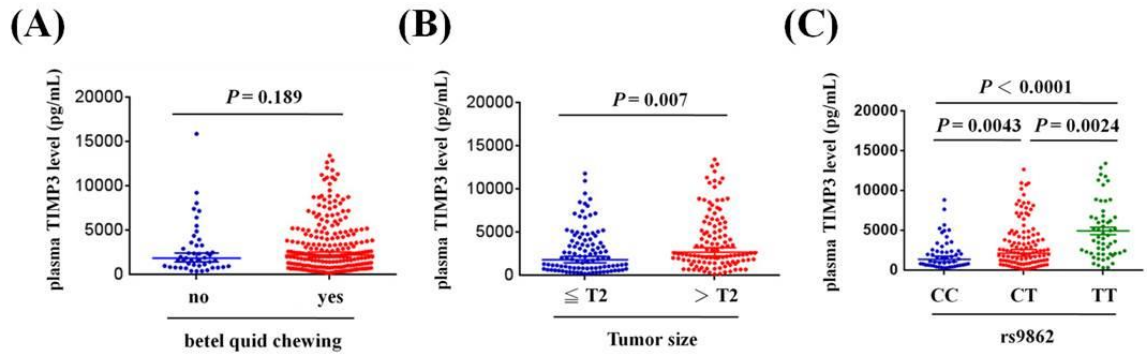

Figure 1. ELISA-determined plasma TIMP3 level of OSCC patients. (A) TIMP3 levels were compared according to betel quid consumption and results showed that TIMP3 levels were increased in betel nut chewers when compared to those without betel quid chewing. (B) Among betel quid chewers, TIMP3 levels were 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.

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

104 年 10 月 22 日

報告人姓名	林巧雯	服務機構及職稱	中山醫學大學口腔科學研究所 助理教授
會議時間 會議地點	2014/06/20-2014/06/23 義大利-佛羅倫斯	本會核定補助文號	
會議名稱	(中文) 2015 歐洲暨美國癌症大會聯合會議 (英文) EACR-AACR-SIC Special Conference 2015		
發表論文題目	(中文) Dehydroandrographolideu 抑制口腔癌細胞的轉移透過影響轉錄因子 SP-1 調控 MMP-2 (英文) Dehydroandrographolide inhibits migration and invasion by inhibition of matrix metalloproteinase-2 through modulation of SP-1 in human oral cancer		

報告內容應包括下列各項：

一、參加會議經過

於 6/19 抵達義大利佛羅倫斯。6/20 前往會議地點(Firenze Fiera) 報到及領取大會議程及摘要手冊，並聆聽演講。6/21 將準備好的論文海報張貼於指定位置。並於會議尚未開始的時間參觀會場內其他相關發表，下午於論文海報張貼處介紹自己的研究成果。

二、與會心得

此次的會議舉辦地點在佛羅倫斯火車站旁的的國際會議中心，在市中心且交通方便，因此參與會議者眾多。會場演講廳可容納 1000 位聽眾。在會場也遇到許多熟識的台大與北醫的教授。而除了在會場中觀摩展示海報發表的研究內容外，也參與多場口頭報告發表，聆聽一些關於 MicroRNA-based therapeutics 與 circulating tumour DNA 的相關研究。藉由參與國際大型會議，可認識國際上頂尖的研究學者，了解大師級的思維，對平日教學與研究頗有助益。

三、建議

國內也應增加舉辦如此大型會議、增加補助出國額度、或盡量補助教師與學生出國開會或短期研究之經費，使學生可以訓練其外語能力與加強國際觀。

四、攜回資料名稱及內容

會議議程手冊

科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/21

科技部補助計畫	計畫名稱: 基質金屬蛋白酶組織抑制因子3在口腔癌致癌過程與表觀遺傳學的研究
	計畫主持人: 林巧雯
	計畫編號: 103-2314-B-040-019- 學門領域: 牙醫學

無研發成果推廣資料

103年度專題研究計畫研究成果彙整表

計畫主持人：林巧雯	**計畫編號：**103-2314-B-040-019-
計畫名稱：基質金屬蛋白酶組織抑制因子3在口腔癌致癌過程與表觀遺傳學的研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	1	100%	人次	碩士生陳姝愉
		博士生	1	1	100%		博士生謝淑卿
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	此篇論文已經接受於Medicine
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文		無					

字敘述填列。）	

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
      □實驗失敗
      □因故實驗中斷
      □其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）