行政院國家科學委員會專題研究計畫 成果報告

Lipocalin-2及其MMP-9複合體在口腔黏膜下纖維化及口腔癌的表現及其機制探討
研究成果報告(精簡版)

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中文摘要： Lipocalin-2(24p3)是Lipocalin蛋白家族的一分子。LCN2可與基質金屬蛋白水解酶(Matrix metalloproteinase-9；MMP-9)結合而促進MMP-9的活性，並且使得MMP-9不被降解。 LCN2除了在人類各種發炎反應會上升，近年來也發現其與腫瘤生成有關。 我們利用酵素聯結免疫吸附法觀察了在口腔鱗狀細胞癌病人(oral squamous cell carcinoma；OSCC)與健康對照組血漿中LCN2、MMP-9與LCN2/MMP-9複合物的表現。結果顯示OSCC病人血漿中LCN2、MMP-9與LCN2/MMP-9複合物的濃度明顯高於健康對照組(LCN2: $p<0.001$, MMP-9: $p<0.001$, LCN2/MMP-9: $p<0.01$)。並且LCN2的表現量與腫瘤大小($p<0.05$)、TNM分期($p<0.05$)有關，但與淋巴轉移、遠端轉移無關。接著使用Spearman ‘s Correlation檢定分析病人血清中LCN2、MMP-9與LCN2/MMP-9複合物濃度之間的相關性。發現這三者指標兩兩皆呈現正相關。

中文關鍵詞： 基質金屬蛋白水解酶、口腔癌、酵素聯結免疫吸附法

英文摘要： Objectives: Recent evidence demonstrated that lipocalin (LCN)2 is induced in many types of human cancer, while detection of its complex with matrix metalloproteinase (MMP)-9 is correlated with the cancer-disease status. We attempted to evaluate plasma expressions of LCN2, MMP-9, and their complex (LCN2/MMP-9) during the diagnostic work-up of patients with oral squamous cell carcinoma (OSCC) and investigated their correlations with disease progression.

Methods: In total, 195 patients with OSCC and 81 healthy controls were recruited. Expression levels of LCN2, MMP-9, and LCN2/MMP-9 were determined with immunoenzymatic assays.

Results: Patients with OSCC exhibited significantly higher levels of LCN2, MMP-9, and LCN2/MMP-9 compared to healthy controls (LCN2: $p<0.001$, MMP-9: $p<0.001$, LCN2/MMP-9: $p<0.01$). Plasma levels of LCN2, MMP-9, and LCN2/MMP-9 in OSCC patients were significantly correlated with each other and were associated with more-advanced clinical stages ($p<0.05$) and/or a larger tumor size ($p<0.05$), but were not associated with positive lymph node metastasis or distal metastasis.

Conclusions: Our results suggest that plasma levels of LCN2 and the LCN2/MMP-9 complex may be useful in non-invasively monitoring OSCC progression, while supporting their potential role as biomarkers of oral cancer disease status.

英文關鍵詞： Lipocalin 2, Matrix metalloproteinase-9, Oral squamous cell carcinoma

The Role of Lipocalin 2 and Its Complex with Matrix Metalloproteinase-9 in Oral Cancer

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Running title: The Role of LCN2 in OSCC

Keywords: Lipocalin 2, Matrix metalloproteinase-9, Oral squamous cell carcinoma

Abstract

Objectives: Recent evidence demonstrated that lipocalin (LCN)2 is induced in many types of human cancer, while detection of its complex with matrix metalloproteinase (MMP)-9 is correlated with the cancer-disease status. We attempted to evaluate plasma expressions of LCN2, MMP-9, and their complex (LCN2/MMP-9) during the diagnostic work-up of patients with oral squamous cell carcinoma (OSCC) and investigated their correlations with disease progression.

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Conclusions: Our results suggest that plasma levels of LCN2 and the LCN2/MMP-9 complex may be useful in non-invasively monitoring OSCC progression, while supporting their potential role as biomarkers of oral cancer disease status.

Introduction

Lipocalin (LCN)2, also known as neutrophil gelatinase-associated lipocalin, neu-related lipocalin, oncogene 24p3, and uterocalin, is a 25-kDa protein which is stored in the granules of human neutrophils (Cowland & Borregaard, 1997). It belongs to the lipocalin family, which comprises more than 50 members, all of which are characterized by the ability to bind and transport small lipophilic substances (Bratt, 2000).

LCN2 participates in iron trafficking (Yang et al., 2002) and increases cytoplasmic levels of this mineral by capturing and transporting iron particles to cell interiors after interacting with specific membrane receptors (24p3 and megalin) (Goetz et al., 2000). Its role in iron delivery to cells underlies the multiple effects attributed to LCN2. Released by activated neutrophils, this protein participates in an iron-depletion strategy exploited by the immune defense against bacterial pathogens (Saiga et al., 2008). In addition, LCN2 seems to be involved in the growth, development, and differentiation of several human tissues, as early as in the embryo, through its regulation of iron-responsive genes which are important in the differentiation of primordial cells (Gwira et al., 2005, Mori et al., 2003). Finally, LCN2 seems to participate in carcinogenesis by favoring iron uptake from extracellular spaces within malignant cells, a fundamental process for maintaining neoplastic cell multiplication (Devireddy et al., 2005). In line with its involvement in carcinogenesis processes, LCN2 synthesis is induced by factors promoting the development of neoplasias (Bratt, 2000, Stoesz et al., 1998), and its overexpression was found in several malignancies including breast (Bauer et al., 2008, Stoesz et al., 1998), gastric (Kubben et al., 2007), esophageal squamous cell (Zhang et al., 2007), colorectal (Nielsen et al., 1996), pancreatic (Furutani et al., 1998, Laurell et al., 2006), lung (Friedl et al., 1999), and

ovarian cancers (Lim et al., 2007). Regarding oral squamous cell carcinoma (OSCC), Hiromoto and coworkers recently investigated the expression levels of LCN2 in oral cancer tissues, found that LCN2 expression is high in well-differentiated cancer, and suggested that LCN2 might be a useful diagnostic marker of tumor-cell differentiation in OSCC (Hiromoto et al., 2011).

It was established that LCN2 forms a complex with matrix metalloproteinase (MMP)-9, thereby preventing MMP-9 autodegradation and increasing its activity *in vitro* (Yan et al., 2001). MMP-9 plays a critical role in cancer progression, invasion, and metastasis in several neoplastic diseases including oral cancer (Choi & Myers, 2008). Since MMP-9 is implicated in both early and late processes of tumor progression through the degradation of the extracellular matrix and basement membranes (Somiari et al., 2006), the question of whether LCN2 and the LCN2/MMP-9 complex contributes to tumor progression was raised. Fernandez et al. investigated the role of the LCN2/MMP-9 complex in breast tumor growth and its presence in the urine of breast-cancer patients (Fernandez et al., 2005). Their findings suggested that detection of the urinary LCN2/MMP-9 complex might represent an independent predictor of the disease status. Recently, Smith and coworkers reported significant elevations in MMP-9 and LCN2/MMP-9 in brain-tumor patients. Their expressions were correlated with the presence of disease and the response to therapy and could be detected in both tumor tissues and urine samples (Smith et al., 2008). An association between the LCN2/MMP-9 complex and gastric cancer was also suggested since expression of the complex in tumor tissues of gastric-cancer patients was highly associated with worse survival and was related to the histological and genetic typing of gastric cancer (Kubben et al., 2007). In comparison to other tumor types, data regarding the correlation between the LCN2/MMP-9 complex and OSCC

progression are still scarce.

To date, most studies focused on LCN2 and LCN2/MMP-9 tissue expressions, while only a few investigated the clinical utility of their urinary levels. LCN2 and MMP-9 are stored in specific granules in neutrophils, while MMP-9 is also found in gelatinase granules. It was suggested that MMP-9 and LCN2 are mainly secreted into the blood by neutrophils infiltrating a tumor, and are subsequently excreted in the urine (Yan et al., 2001). Although detecting LCN2 and its complex with MMP-9 in systemic circulation seems reasonable, few studies on LCN2 and LCN2/MMP-9 in plasma are currently available (Haase-Fielitz et al., 2009, Tsai et al., 2011). The aim of our study was to evaluate plasma levels of LCN2, MMP-9, and the LCN2/MMP-9 complex in patients with OSCC and investigate their correlations with OSCC progression.

Materials and Methods

Subjects and specimen collection

We recruited 195 patients (189 males and 6 females with a mean age of 53.56 ± 11.43 years) at Chung Shan Medical University Hospital in Taichung and Changhua Christian Hospital in Changhua, Taiwan as a case group between 2008 and 2011. For a control group, we randomly chose 81 non-cancer individuals (77 males and 4 females with a mean age of 51.16 ± 11.52 years) who visited those same hospitals and were from the same geographic area. For both cases and controls, we used a questionnaire to obtain exposure information about betel quid chewing and tobacco use. Medical information on the cases, including TMN clinical staging, primary tumor size, lymph-node involvement, and histological grade, was obtained from their medical records. Oral-cancer patients were clinically staged at the time of diagnosis according to the TNM staging system of the *American Joint Committee on Cancer (AJCC) Staging Manual,* seventh edition. Tumor differentiation was examined by a pathologist according to the AJCC classification. Whole-blood specimens collected from the controls and OSCC patients were placed in tubes containing ethylenediaminetetraacetic acid (EDTA), immediately centrifuged, and stored at -80 °C. Before conducting this study, approval was received from the Institutional Review Board of Chung Shan Medical University Hospital, and informed written consent to participate in the study was obtained from each individual.

Measurements of plasma LCN2, MMP-9, and LCN2/MMP-9 levels by an enzyme-linked immunosorbent assay (ELISA)

LCN2, MMP-9, and LCN2/MMP-9 levels in plasma samples were respectively analyzed using human LCN2, MMP-9, and LCN2/MMP-9 ELISA kits (R&D Systems,

Abingdon, UK). From each plasma sample, 100 μL was directly transferred to the microtest strip wells of the ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microtest plate spectrophotometer, and LCN2, MMP-9, and LCN2/MMP-9 levels were quantified with a calibration curve using human LCN2, MMP-9, and LCN2/MMP-9 as standards.

Statistical Analysis

The Mann-Whitney U-test was used to test for statistical significance of differences in LCN2, MMP-9, and LCN2/MMP-9 between the 195 patients with OSCC and the 81 normal controls. Least squares means were calculated to predict the adjusted LCN2, MMP-9, and LCN2/MMP-9 levels for our study subjects with different betel quid chewing status and smoking habits. Correlations of LCN2, MMP-9, and LCN2/MMP-9 with clinicopathologic parameters of OSCC were examined by Pearson's χ^2 test. Spearman's rank correlation analysis was used to evaluate the correlations of these parameters. The SPSS statistical package vers. 12.0 (SPSS, Chicago, IL) was used for the statistical analysis. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics

The study included two different study groups, OSCC patients and healthy control subjects. Table 1 presents the demographic data, which showed that 11 of 81 healthy control subjects (13.6%) and 151 of 195 patients with oral cancer (77.4%) had chewed betel nuts, and 37 of 81 healthy control subjects (45.7%) and 158 of 195 patients with oral cancer (81.0%) had smoked. The distributions of betel nut chewing ($p<0.001$), and tobacco consumption ($p<0.001$) between healthy control subjects and patients with oral cancer significantly differed.

Plasma Levels and Correlations of LCN2, MMP-9, and LCN2/MMP-9 Complex in Different Study Groups

Table 1 also presents the mean plasma levels of LCN2, MMP-9, and LCN2/MMP-9 complex in healthy control subjects and patients with OSCC. Patients with OSCC exhibited significantly increased levels of all three molecules compared to healthy control subjects (LCN2: $p=0.001$, MMP-9: $p<0.001$, LCN2/MMP-9: $p= 0.032$) after adjusting for betel nut chewing and smoking habits. Among the 195 patients with OSCC, significant positive correlations between plasma levels of LCN2/MMP-9 and LCN2 and between LCN2/MMP-9 and MMP-9 were observed (Spearman's rank correlation coefficient $\gamma=0.362$, $p<0.0001$; $\gamma=0.717$, $p<0.0001$, respectively; Figure 1A, B). Moreover, a significant correlation was also found between plasma levels of LCN2 and MMP-9 in OSCC patients (Spearman's rank correlation coefficient $\gamma=0.223$, $p=0.0018$; Figure 1C).

Associations of Plasma LCN2, MMP-9, and LCN2/MMP-9 Levels with Clinical

Parameters of Patients and Histopathological Properties of the Tumor

Table 2 presents associations of LCN2, MMP-9, and LCN2/MMP-9 with clinicopathological parameters. A higher plasma level of LCN2 was significantly associated with a more-advanced TNM stage (p=0.035) and a larger tumor size (p=0.009). The mean level of plasma LCN2 in T1 or T2 status was significantly lower than that in T4 status (p=0.012 and 0.046, respectively; Figure 2A), and the LCN2 level in stage I or II was significantly lower than that in stage IV (p=0.032 and 0.042, respectively; Figure 2B). In addition, a higher plasma level of LCN2 was more prevalent in the older group (≥52 y) of OSCC patients (p=0.036). Furthermore, plasma MMP-9 levels were positively correlated to advanced TNM stage (p=0.041), while plasma LCN2/MMP-9 complex levels to tumor size (p=0.034). Moreover, no significant association was found among these three molecules with patient gender, smoking status, betel nut chewing habit, lymph node metastasis, distal metastasis, or the tumor differentiation status (Table 2).

Discussion

This is, to the best of our knowledge, the first study to investigate peripheral blood levels and potential roles of LCN2, MMP-9, and the LCN2/MMP-9 complex in OSCC. In this study, we found that plasma levels of these three molecules in patients with OSCC were higher compared to those in healthy controls. Moreover, plasma levels of these three molecules in OSCC patients were significantly correlated with each other and were associated with more-advanced clinical stages and/or a larger tumor size.

Elevated levels of LCN2 expression were detected in several kinds of human tumors (Bauer et al., 2008, Friedl et al., 1999, Furutani et al., 1998, Laurell et al., 2006, Stoesz et al., 1998). Lim et al. reported that tissue expression of LCN2 in ovarian tumors changes with the disease grade, and this is also reflected in serum levels (Lim et al., 2007). The role of LCN2 was also investigated in esophageal squamous cell carcinoma (ESCC), and it was reported that its tissue expression was significantly higher in ESCC than in normal mucosa, and was positively correlated with cell differentiation (Zhang et al., 2007). Recently, it was also demonstrated that the expression of LCN2 in OSCC tissue was significantly correlated with the cancer-cell differentiation status and morphological patterns (epithelial-mesenchymal transition, EMT) (Hiromoto et al., 2011). In Hiromoto's study, they analyzed LCN2 expression by IHC from 30 OSCC specimens and found that LCN2 is predominantly expressed in well-differentiated OSCC tissues, with lower expression in moderately or poorly differentiated OSCC tissues. The staining scores differed significantly between well-differentiated and moderately differentiated OSCC, as well as between well-differentiated and poorly differentiated OSCC. However, the significant correlation between LCN2 expression level and OSCC differentiated status can not be

observed in our study. This discrepancy might be due to the different sample size, specimen source, and methods used for LCN2 detection.

It was reported that LCN2 can prevent MMP-9 degradation, increasing its activity by binding and forming the LCN2/MMP-9 complex. Tumor cells excrete elevated levels of LCN2 resulting in an increase in the local concentration of MMP-9, which can affect various aspects of tumor progression (Yan et al., 2001). Moreover, it was reported that tumor cell-secreted MMP-9 promotes angiogenesis *in vivo* in a breast-cancer model where the vascular endothelial growth factor (VEGF)/VEGF receptor 2 association was shown to be dependent on MMP-9 activity (Mira et al., 2004). In oral cancer, previous reports showed a good correlation between increased tumor vascularity and tumor progression in OSCC (Alcalde et al., 1995, Macluskey et al., 2000, Williams et al., 1994). Previous reports also indicated that VEGF is a vital regulator of OSCC tumor angiogenesis, as evidenced by the high expression level of VEGF in oral cancerous tissues and several OSCC cell lines (Shintani et al., 2004) and the angiogenic inhibition potential of the soluble form of the VEGF 2 receptor *in vivo* (Okada et al., 2010). In this study, we found a significant correlation between plasma levels of LCN2 and LCN2/MMP-9 in patients with OSCC. We further found that plasma levels of LCN2 and the LCN2/MMP-9 complex in OSCC patients were significantly correlated with a larger tumor size. We propose that regulation of OSCC tumor growth by LCN2 might be through increased MMP-9 activity which further promotes VEGF-mediated angiogenic regulation. However, in this study, we did not determine if the higher expression of LCN2 was associated with the often highly vascular OSCC specimens or the role of LCN2 in tumor angiogenesis of OSCC. In our future work, we will collect plasma and tumor tissues from OSCC patients, and further validation studies with LCN2 and the endothelial cell marker, CD 31,

antibodies should be conducted to characterize the role of LCN2 in angiogenesis using a larger cohort of OSCC specimens.

In addition to tumor growth regulation, recent evidence suggests that LCN2 expression is associated with the progression of cancer invasion. For example, it was reported that LCN2 serum levels were significant higher in patients with invasive breast cancer while moderate in patients with ductal carcinoma in situ, atypical ductal hyperplasia, and sclerosing adenosis compared to healthy controls (Provatopoulou et al., 2009). Moreover, knockdown of LCN2 in cholangiocarcinoma cells was reported to suppress their invasive ability through reducing LCN2/MMP-9 complex formation (Nuntagowat et al., 2010). Overexpression of LCN2 can promote the invasive ability of colon carcinoma cells through activating Rac1 signaling (Hu et al., 2009). In contrast to promoting invasion, LCN2 was also reported to inhibit adhesion and invasion in pancreatic cancer, suggesting that LCN2 may be a suppressor of pancreatic cancer progression (Tong et al., 2008).

Limitations of this study are a small sample size, and the age and gender not being well matched between the controls and cases. In a future study, we will increase of specimen number and take more OSCC risk factors into account in the analysis to see if we can precisely validate these findings. Additionally, the functional role of LCN2 in OSCC growth or invasion is worth further investigation. We will establish *LCN2* knockdown clones to elucidate the possible function of LCN2 (proliferation, cell cycle regulation, angiogenesis, migration, invasion, EMT) in OSCC cell lines, and further investigate their underlying mechanisms. Moreover, the *LCN2* knockdown clones will be applied for the different animal models to investigate the role of LCN2 on tumor growth and metastasis *in vivo* in our future work.

Improved non-operative diagnostic techniques enable the detection of oral cancer

at an earlier stage. At the same time, serum or plasma measurements represent a non-invasive, easily accessible method for the study of biomarkers as screening tools for risk assessment, diagnosis, and prognosis of oral cancer. The present study was designed in an effort to reveal specific biomarkers associated with OSCC progression. Our data suggest that plasma LCN2 and the LCN2/MMP-9 complex are positively correlated with OSCC progression, with potential clinical utility as biomarkers of the OSCC status.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Author contributions

CW Lin and MH Chien designed the study and prepared the manuscript. SF Yang, SW Tseng and CH Lin analyzed the data. YS Hsieh, CP Ko, LH Wei, CW Lin, and MH Chien performed the study plan and collected tissues.

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Table 1. Demographic characteristics and clinical features of oral squamous cell carcinoma (OSCC) patients and healthy controls

Variables	OSCC patients n=195	Controls n=81	p value
Age (years)	53.56 ± 11.43 [a]	51.16 ± 11.52	0.114
Gender: male (%)	189 (99.0%)	77 (95.1%)	0.102
Betel nuts chewing			
Yes	151 (77.4%)	11 (13.6%)	<0.001
No	44 (22.6%)	70 (86.4%)	
Smoking status			
Yes	158 (81.0%)	37 (45.7%)	<0.001
No	37 (19.0%)	44 (54.3%)	
LCN2 (ng/mL)	51.12 ± 2.37	34.88 ± 3.99	0.001 [b]
MMP-9 (ng/mL)	122.63 ± 5.19	68.03 ± 8.80	<0.001 [b]
LCN2/MMP-9 complex (ng/mL)	45.17 ± 2.72	32.68 ± 4.60	0.032 [b]
Tumor T status			
T1	58 (29.7%)	-	
T2	61 (31.3%)	-	
T3	26 (13.3%)	-	
T4	50 (25.6%)	-	
Lymph node status			
N0	137 (70.3%)	-	
N1	30 (15.4%)	-	
N2	27 (13.8%)	-	
N3	1 (0.5%)	-	
Metastasis			
M0	193 (99.0%)	-	
M1	2 (1.0%)	-	
Cell differentiation			
Well differentiated	23 (11.8%)	-	
Moderately or poorly differentiated	172 (88.2%)	-	
Stage			
I	52 (26.7%)		
II	42 (21.5%)		
III	33 (16.9%)		
IV	68 (34.9%)		

[a] Mean ± standard deviation.

[b] After adjusting for betel nut chewing and smoking habits.

Table 2. Correlations of lipocalin (LCN)2, matrix metalloproteinase (MMP)-9, and the LCN2/MMP-9 complex plasma levels with clinicopathological parameters in 195 oral squamous cell carcinoma (OSCC) patients

Variables	LCN2 (ng/mL)	*p* value	MMP-9 (ng/mL)	*p* value	LCN2/MMP-9 complex (ng/mL)	*p* value
	n=195		*n*=195		*n*=195	
Age (years)						
<52.0 (*n*=90)	45.47 ± 31.91	0.036	126.66 ± 81.90	0.523	49.69 ± 51.48	0.191
≥52.0 (*n*=105)	56.01 ± 36.98		119.69 ± 70.10		42.13 ± 26.75	
Gender						
male (*n*=192)	28.57 ± 10.71	0.262	124.43 ± 56.54	0.972	46.75 ± 5.89	0.961
Female (*n*=3)	51.50 ± 35.18		122.88 ± 76.03		45.60 ± 40.48	
Smoking status						
No (*n*=37)	46.26 ± 27.70	0.348	118.58 ± 76.91	0.700	42.93 ± 36.70	0.652
Yes (*n*=158)	52.29 ± 36.53		123.92 ± 75.57		46.25 ± 41.03	
Betel nut chewing						
No (*n*=44)	50.04 ± 31.89	0.813	124.23 ± 64.93	0.895	43.59 ± 23.89	0.705
Yes (*n*=151)	51.47 ± 36.00		122.52 ± 78.70		46.21 ± 43.84	
Tumor T status						
T1+T2 (*n*=119)	45.93 ± 28.19	0.009*	115.33 ± 66.42	0.080	40.75 ± 24.49	0.034*
T3+T4 (*n*=76)	59.30 ± 42.61		134.78 ± 87.36		53.24 ± 55.98	
Lymph node status						
N0 (*n*=137)	49.70 ± 35.47	0.376	116.80 ± 67.44	0.083	43.48 ± 31.02	0.255
N1+N2+N3 (*n*=58)	54.57 ± 34.06		137.34 ± 91.25		50.67 ± 56.21	
Metastasis						
M0 (*n*=193)	50.87 ± 34.79	0.275	122.25 ± 75.25	0.236	45.32 ± 40.22	0.311
M1 (*n*=2)	78.13 ± 65.81		186.17 ± 123.18		74.33 ± 29.69	
Stage						
I+II (*n*=94)	45.68 ± 27.48	0.035*	111.46 ± 64.46	0.041*	40.23 ± 24.27	0.071
III+IV (*n*=101)	56.23 ± 40.32		133.57 ± 86.68		50.62 ± 50.30	
Cell differentiation						
Well differentiated (*n*=23)	46.01 ± 27.29	0.456	115.07 ± 70.90	0.598	41.64 ± 27.69	0.615
Moderately or poorly differentiated (*n*=172)	51.83 ± 35.96		123.96 ± 76.41		46.15 ± 41.59	

* $p<0.05$.

Figure legends

Figure 1. Correlations among plasma lipocalin (LCN)2, matrix metalloproteinase (MMP)-9 and the LCN2/MMP-9 complex expressions in 195 oral squamous cell carcinoma (OSCC) patients. (A) There was a significant correlation between plasma LCN2 and the LCN2/MMP-9 complex (Spearman correlation coefficients $\gamma=0.362$, $p<0.0001$) in the 195 OSCC patients. (B) There was a significant correlation between plasma MMP-9 and the LCN2/MMP-9 complex (Spearman correlation coefficients $\gamma=0.717$, $p<0.0001$) in the 195 OSCC patients. (C) There is a significant correlation between plasma LCN2 and MMP-9 levels (Spearman correlation coefficients $\gamma=0.223$, $p=0.0018$) in the 195 OSCC patients.

Figure 2. Comparison of plasma lipocalin (LCN)2 expression with various tumor sizes and at various stages in 195 OSCC patients. (A) LCN2 levels were compared according to the tumor size and results showed that LCN2 levels at T4 significantly differed between those at T1 and T2. (B) LCN2 levels were compared according to the stage, and results showed that LCN2 levels in stage IV significantly differed from those at stages I and II.

Figure 1A

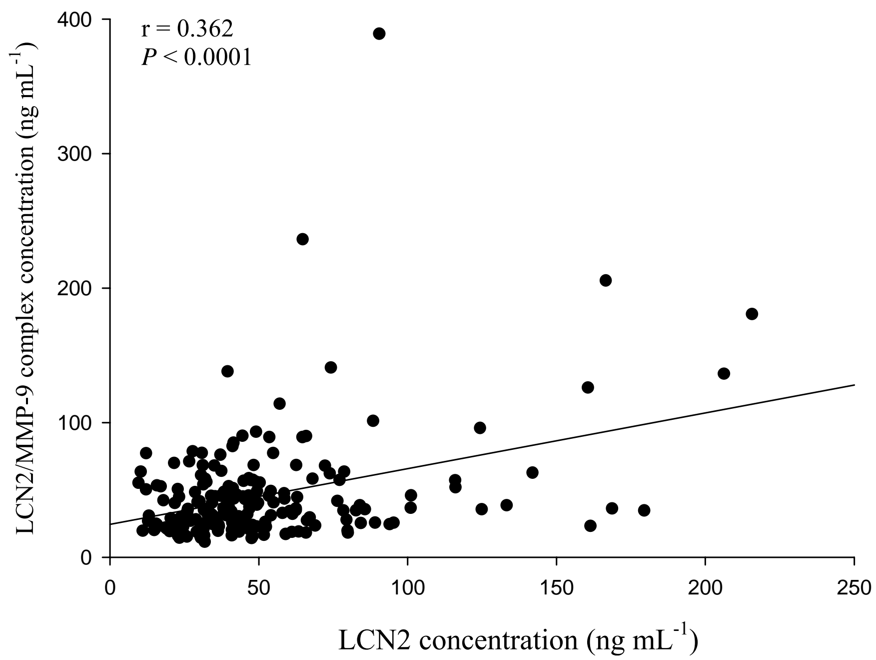


Figure 1B

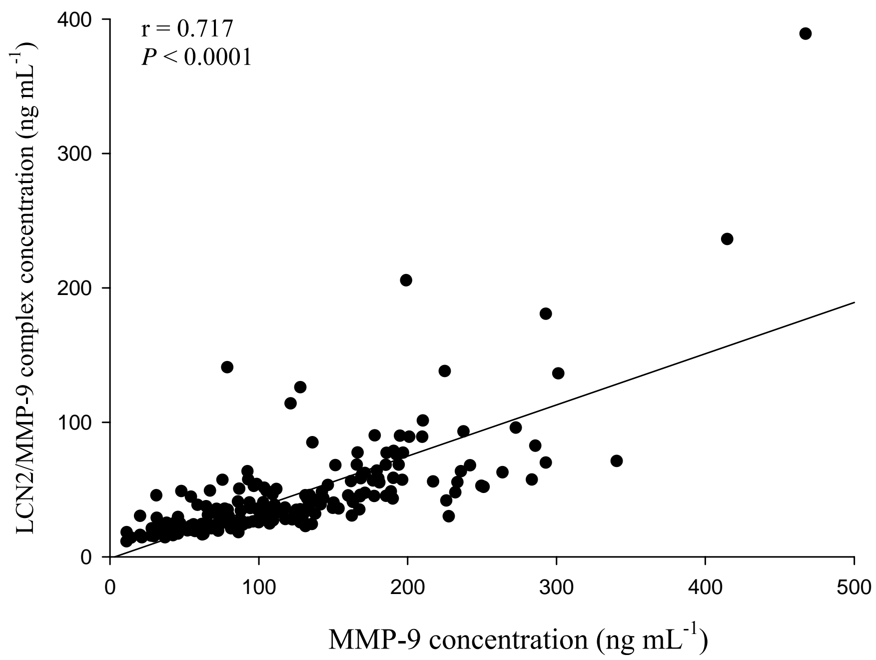


Figure 1C

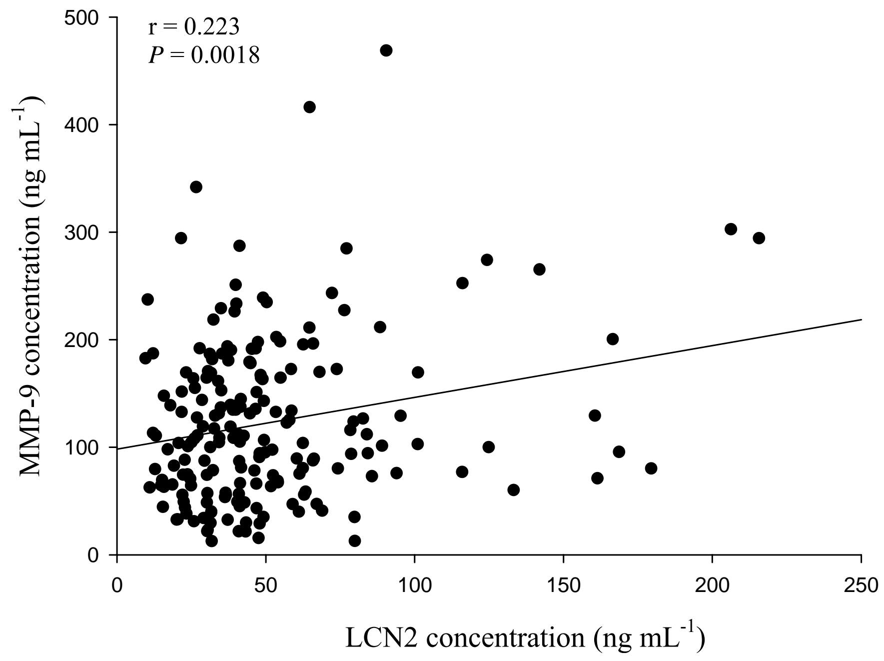


Figure 2A

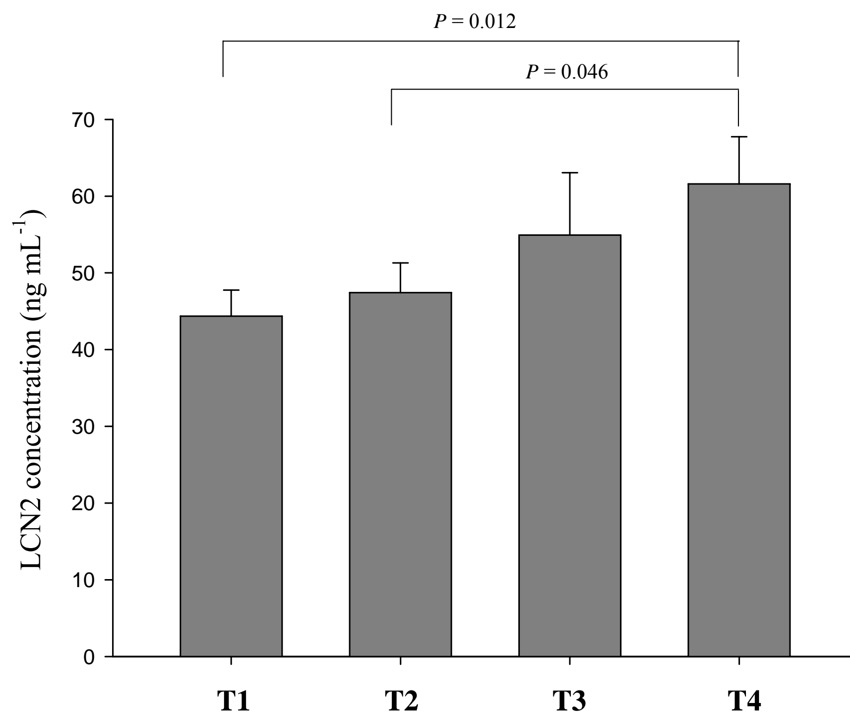
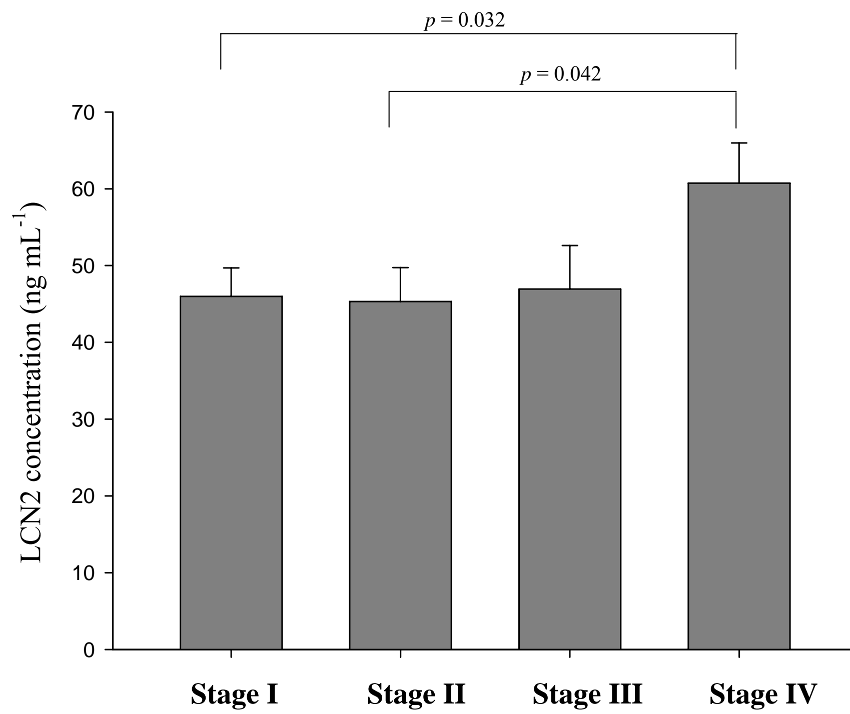


Figure 2B



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

101 年 10 月 28 日

報告人姓名	楊順發	服務機構及職稱	中山醫學大學 醫學研究所 教授
會議 時間 地點	2012/06/20~2012/06/23 福斯多伊瓜蘇（巴西 ）	本會核定 補助文號	
會議名稱	（中文）第 90 屆國際聯合牙醫研究會議 （英文）90 th General Session & Exhibition of the International Association for Dental Research		
發表論文題目	（中文）Lipocalin 2 其複合物在口腔癌的相關性探討 （英文）Lipocalin 2 and Its Complex with MMP-9 in Oral cancer		

報告內容應包括下列各項：

一、參加會議經過

6/21 抵達伊瓜蘇開會地點，6/22 早上前往 Centro de Convenções 報到及領取大會議程及摘要手冊，並張貼研究成果海報，之後參觀其他研討成果展覽以及聆聽多場會議報告。下午則在成果海報處講解研究內容。6/23 再度前往會場與其他參加者交流研究報告。

二、與會心得

本人的研究主題主要是 Lipocalin 2 其複合物在口腔癌的相關性探討，而本次大會的主題除了涵蓋人類口腔癌的基礎研究之外，還加入臨床治療及個案探討，因此藉由此次會議讓我有機會接觸到更實際的臨床領域，獲得不少新觀念及之前未曾有過的一些想法。會中聆聽許多大師級的演講，受益良多。與其他相關研究人員的諸多討論，也獲得很多寶貴的意見及肯定。此類與國外研究人員的溝通及聯繫是很重要的，讓我有機會與國外實驗室有初步之合作構想，並已有初步之計畫，希望能藉此有國際合作的機會。

三、建議

國內應多加舉辦如此大型會議、增加補助出國額度、或盡量補助博士班學生出國開會或短期研究之經費，讓年輕研究學者有機會與大師級學者學習。

四、攜回資料名稱及內容

會議議程手冊
會議摘要手冊

Friday, March 23, 2012
Shun-Fa Yang
Chung Shan Medical University
Taichung, Taiwan
Abstract ID#: 162454
Abstract Title: Lipocalin 2 and Its Complex with MMP-9 in Oral cancer
Dear Shun-Fa Yang,
It is a pleasure to inform you that your abstract has been ACCEPTED as a POSTER PRESENTATION at the IADR General Session (June 20-23, 2012). The meeting will take place in Igua蓷 Falls, Brazil.
Please note that some students/co-workers have provided an alternate e-mail address for notification, so if this letter is addressed to a colleague, please forward it to his/her attention. E-mail notifications are sent only to the address provided for the presenter when the abstract was submitted; it is the presenter씜 responsibility to notify co-authors.
Please DO NOT lose this notification. The mode of your presentation has been assigned by the Group Program Chair and must be followed as we are unable to change it at this date. Assignments were based on authors' requests as much as possible. Please note that your final presentation number will be assigned next month (see note below for details).

PRESENTATION INFORMATION
Presentation Date: Friday, June 22, 2012
Session Title: Carcinogenesis
Session Time: 2:15 PM - 3:30 PM
Location: Convention Center, Poster Hall

Poster Viewing Time: 9 a.m. ?5 p.m.
Poster Set-up Time: 8:30 a.m. ?9 a.m.
Poster Tear-down Time: 5 p.m. ?5:15 pm

POSTER SIZE
Dimensions of the poster board are 1m x 1.98m (3.28 ft x 6.5 ft). The board will be used VERTICALLY. These are the maximum dimensions to follow when creating your poster but you may make your poster smaller. You are only required to be at your poster board during the session time listed above and not the entire poster viewing time. For further information please go directly to the meeting home page on the IADR website at www.iadr.org/iags.

POSTER SUPPLIES

Please make every attempt to bring your own tape or double-sided velcro to mount your poster as a limited supply will be available.

PRE-REGISTRATION REQUIREMENT

All presenters must pre-register and pay the applicable fee by the April 20, 2012 presenter deadline. Approximately one week after the presenter registration deadline, you will be sent a notification confirming your participation with your final presentation and sequence numbers (the notices will be sent to all presenters at once after the registration deadline and not sent individually after you register). If you do not pre-register, you will NOT be allowed to participate in the meeting and your abstract will be withdrawn from the final printed Program Book and will not be citable as appearing in the special edition of the Journal for Dental Research. If you need an invitation letter to get a visa, please check the applicable box when registering and a letter will be sent to you.

IMPORTANT MEETING LINKS

Program Book Listing:

Your title will be printed in the Program Book as submitted here:

Lipocalin 2 and Its Complex with MMP-9 in Oral cancer

If you would like to edit your presentation title (not to exceed 10 words), please visit the link below by April 6, 2012 and use your assigned Abstract ID 162454 and password 541325:

http://iadr.confex.com/iadr/extra.cgi?username=162454&password=541325&EntryType=Paper

To register for the meeting:

http://www.iadr.org/i4a/pages/index.cfm?pageID=3997

You will need your Abstract ID# to register for the meeting: 162454

Online Scientific Program:

http://www.iadr.org/iags

To reserve a hotel room:

http://www.iadr.org/i4a/pages/index.cfm?pageID=3999

Important travel and visa requirements (note: many countries such as Australia, India, Japan, Nigeria and the United States require a visa to travel to Brazil).
http://www.iadr.org/i4a/pages/index.cfm?pageid=3990

For full details on the new General Session format please visit
http://www.iadr.org/i4a/pages/index.cfm?pageID=4007.

To find other important meeting information, presenter guidelines, new initiatives, featured presentations etc., please visit the meeting home page:
http://www.iadr.org/iags

If you have arranged your travel already and you arrive later or depart earlier than your assigned presentation time, we will not be able to move your presentation to accommodate your travel plans.

Thank you for submitting your paper. We look forward to your presentation at the meeting and note that we have scheduled a full conference so we hope you stay for the duration of the meeting. If you have any questions, please send a message to meetings@iadr.org. Every attempt will be made to respond as soon as possible.

Sincerely,

Mary MacDougall, PhD
IADR Annual Session Committee Chair

Lipocalin 2 and Its Complex with MMP-9 in Oral cancer

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Abstract:

Objectives: Recent evidence demonstrates that lipocalin 2 (LCN2) is induced in many types of human cancer, while detection of its complex with matrix metalloproteinase-9 (MMP-9) is correlated with cancer disease status. We aim to evaluate the plasma expression of LCN2, MMP-9, and their complex (LCN2/MMP-9) during the diagnostic work-up of patients with oral squamous cell carcinoma (OSCC) and investigate their correlation with disease severity.

Methods: A total of 195 patients with OSCC and 81 healthy controls were recruited. Expression levels of LCN2, MMP-9, and their complex LCN2/MMP-9 were determined with immunoenzymatic assays.

Results: Patients with OSCC exhibited significantly increased levels of LCN2, MMP-9, and LCN2/MMP-9 compared to healthy controls (LCN2: $p < 0.001$, MMP-9: $p < 0.001$, LCN2/MMP-9: $p < 0.01$). The plasma levels of LCN2, MMP-9, and LCN2/MMP-9 in OSCC patients were significantly correlated with each other and were associated with more-advanced clinical stages ($p < 0.05$) and/or a larger tumor size ($p < 0.05$), but were not associated with positive lymph node metastasis or distal metastasis.

Conclusions: Our results suggest that the plasma measurement of LCN2 and MMP-9 may be useful in non-invasively monitoring OSCC progression, while supporting their potential role as biomarkers of oral cancer disease status.

Introduction

Lipocalin (LCN)2, also known as neutrophil gelatinase-associated lipocalin, neu-related lipocalin, oncogene 24p3, and uterocalin, is a 25-kDa protein which is stored in the granules of human neutrophils (Cowland & Borregaard, 1997). It belongs to the lipocalin family, which comprises more than 50 members, all of which are characterized by the ability to bind and transport small lipophilic substances (Bratt, 2000).

LCN2 participates in iron trafficking (Yang et al., 2002) and increases cytoplasmic levels of this mineral by capturing and transporting iron particles to cell interiors after interacting with specific membrane receptors (24p3 and megalin) (Goetz et al., 2000). Its role in iron delivery to cells underlies the multiple effects attributed to LCN2. Released by activated neutrophils, this protein participates in an iron-depletion strategy exploited by the immune defense against bacterial pathogens (Saiga et al., 2008). In addition, LCN2 seems to be involved in the growth, development, and differentiation of several human tissues, as early as in the embryo, through its regulation of iron-responsive genes which are important in the differentiation of primordial cells (Gwira et al., 2005, Mori et al., 2003). Finally, LCN2 seems to participate in carcinogenesis by favoring iron uptake from extracellular spaces within malignant cells, a fundamental process for maintaining neoplastic cell multiplication (Devireddy et al., 2005). In line with its involvement in carcinogenesis processes, LCN2 synthesis is induced by factors promoting the development of neoplasias (Bratt, 2000, Stoesz et al., 1998), and its overexpression was found in several malignancies including breast (Bauer et al., 2008, Stoesz et al., 1998), gastric (Kubben et al., 2007), esophageal squamous cell (Zhang et al., 2007), colorectal (Nielsen et al., 1996), pancreatic (Furutani et al., 1998, Laurell et al., 2006), lung (Friedl et al., 1999), and

ovarian cancers (Lim et al., 2007). Regarding oral squamous cell carcinoma (OSCC), Hiromoto and coworkers recently investigated the expression levels of LCN2 in oral cancer tissues, found that LCN2 expression is high in well-differentiated cancer, and suggested that LCN2 might be a useful diagnostic marker of tumor-cell differentiation in OSCC (Hiromoto et al., 2011).

It was established that LCN2 forms a complex with matrix metalloproteinase (MMP)-9, thereby preventing MMP-9 autodegradation and increasing its activity *in vitro* (Yan et al., 2001). MMP-9 plays a critical role in cancer progression, invasion, and metastasis in several neoplastic diseases including oral cancer (Choi & Myers, 2008). Since MMP-9 is implicated in both early and late processes of tumor progression through the degradation of the extracellular matrix and basement membranes (Somiari et al., 2006), the question of whether LCN2 and the LCN2/MMP-9 complex contributes to tumor progression was raised. Fernandez et al. investigated the role of the LCN2/MMP-9 complex in breast tumor growth and its presence in the urine of breast-cancer patients (Fernandez et al., 2005). Their findings suggested that detection of the urinary LCN2/MMP-9 complex might represent an independent predictor of the disease status. Recently, Smith and coworkers reported significant elevations in MMP-9 and LCN2/MMP-9 in brain-tumor patients. Their expressions were correlated with the presence of disease and the response to therapy and could be detected in both tumor tissues and urine samples (Smith et al., 2008). An association between the LCN2/MMP-9 complex and gastric cancer was also suggested since expression of the complex in tumor tissues of gastric-cancer patients was highly associated with worse survival and was related to the histological and genetic typing of gastric cancer (Kubben et al., 2007). In comparison to other tumor types, data regarding the correlation between the LCN2/MMP-9 complex and OSCC

progression are still scarce.

To date, most studies focused on LCN2 and LCN2/MMP-9 tissue expressions, while only a few investigated the clinical utility of their urinary levels. LCN2 and MMP-9 are stored in specific granules in neutrophils, while MMP-9 is also found in gelatinase granules. It was suggested that MMP-9 and LCN2 are mainly secreted into the blood by neutrophils infiltrating a tumor, and are subsequently excreted in the urine (Yan et al., 2001). Although detecting LCN2 and its complex with MMP-9 in systemic circulation seems reasonable, few studies on LCN2 and LCN2/MMP-9 in plasma are currently available (Haase-Fielitz et al., 2009, Tsai et al., 2011). The aim of our study was to evaluate plasma levels of LCN2, MMP-9, and the LCN2/MMP-9 complex in patients with OSCC and investigate their correlations with OSCC severity.

Materials and Methods

Subjects and specimen collection

We recruited 195 patients (189 males and 6 females with a mean age of 53.56 ± 11.43 years) at Chung Shan Medical University Hospital in Taichung and Changhua Christian Hospital in Changhua, Taiwan as a case group between 2008 and 2011. For a control group, we randomly chose 81 non-cancer individuals (77 males and 4 females with a mean age of 51.16 ± 11.52 years) who visited those same hospitals and were from the same geographic area. For both cases and controls, we used a questionnaire to obtain exposure information about betel quid chewing and tobacco use. Medical information on the cases, including TMN clinical staging, primary tumor size, lymph-node involvement, and histological grade, was obtained from their medical records. Oral-cancer patients were clinically staged at the time of diagnosis according to the TNM staging system of the *American Joint Committee on Cancer (AJCC) Staging Manual,* seventh edition. Tumor differentiation was examined by a pathologist according to the AJCC classification. Whole-blood specimens collected from the controls and OSCC patients were placed in tubes containing ethylenediaminetetraacetic acid (EDTA), immediately centrifuged, and stored at -80 °C. Before conducting this study, approval was received from the Institutional Review Board of Chung Shan Medical University Hospital, and informed written consent to participate in the study was obtained from each individual.

Measurements of plasma LCN2, MMP-9, and LCN2/MMP-9 levels by an enzyme-linked immunosorbent assay (ELISA)

LCN2, MMP-9, and LCN2/MMP-9 levels in plasma samples were respectively analyzed using human LCN2, MMP-9, and LCN2/MMP-9 ELISA kits (R&D Systems,

Abingdon, UK). From each plasma sample, 100 μL was directly transferred to the microtest strip wells of the ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microtest plate spectrophotometer, and LCN2, MMP-9, and LCN2/MMP-9 levels were quantified with a calibration curve using human LCN2, MMP-9, and LCN2/MMP-9 as standards.

Statistical Analysis

The Mann-Whitney U-test was used to test for statistical significance of differences in LCN2, MMP-9, and LCN2/MMP-9 between the 195 patients with OSCC and the 81 normal controls. Least squares means were calculated to predict the adjusted LCN2, MMP-9, and LCN2/MMP-9 levels for our study subjects with different betel quid chewing status and smoking habits. Correlations of LCN2, MMP-9, and LCN2/MMP-9 with clinicopathologic parameters of OSCC were examined by Pearson's χ^2 test. Spearman's rank correlation analysis was used to evaluate the correlations of these parameters. The SPSS statistical package vers. 12.0 (SPSS, Chicago, IL) was used for the statistical analysis. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics

The study included two different study groups, OSCC patients and healthy control subjects. Table 1 presents the demographic data, which showed that 11 of 81 healthy control subjects (13.6%) and 151 of 195 patients with oral cancer (77.4%) had chewed betel nuts, and 37 of 81 healthy control subjects (45.7%) and 158 of 195 patients with oral cancer (81.0%) had smoked. The distributions of betel nut chewing (p<0.001), and tobacco consumption (p<0.001) between healthy control subjects and patients with oral cancer significantly differed.

Plasma Levels and Correlations of LCN2, MMP-9, and LCN2/MMP-9 Complex in Different Study Groups

Table 1 also presents the mean plasma levels of LCN2, MMP-9, and LCN2/MMP-9 complex in healthy control subjects and patients with OSCC. Patients with OSCC exhibited significantly increased levels of all three molecules compared to healthy control subjects (LCN2: p=0.001, MMP-9: p<0.001, LCN2/MMP-9: p= 0.032) after adjusting for betel nut chewing and smoking habits. Among the 195 patients with OSCC, significant positive correlations between plasma levels of LCN2/MMP-9 and LCN2 and between LCN2/MMP-9 and MMP-9 were observed (Spearman's rank correlation coefficient γ=0.362, p<0.0001; γ=0.717, p<0.0001, respectively; Figure 1A, B). Moreover, a significant correlation was also found between plasma levels of LCN2 and MMP-9 in OSCC patients (Spearman's rank correlation coefficient γ=0.223, p=0.0018; Figure 1C).

Associations of Plasma LCN2, MMP-9, and LCN2/MMP-9 Levels with Clinical

Parameters of Patients and Histopathological Properties of the Tumor

Table 2 presents associations of LCN2, MMP-9, and LCN2/MMP-9 with clinicopathological parameters. A higher plasma level of LCN2 was significantly associated with a more-advanced TNM stage (p=0.035) and a larger tumor size (p=0.009). The mean level of plasma LCN2 in stage T1 or T2 was significantly lower than that in stage T4 (p=0.012 and 0.046, respectively; Figure 2A), and the LCN2 level in stage I or II was significantly lower than that in stage IV (p=0.032 and 0.042, respectively; Figure 2B). In addition, a higher plasma level of LCN2 was more prevalent in the older group (≥52 y) of OSCC patients (p=0.036). Furthermore, plasma MMP-9 levels were positively correlated to advanced TNM stage (p=0.041), while plasma LCN2/MMP-9 complex levels to tumor size (p=0.034). Moreover, no significant association was found among these three molecules with patient gender, smoking status, betel nut chewing habit, lymph node metastasis, distal metastasis, or the tumor differentiation status (Table 2).

Discussion

This is, to the best of our knowledge, the first study to investigate peripheral blood levels and potential roles of LCN2, MMP-9, and the LCN2/MMP-9 complex in OSCC. In this study, we found that plasma levels of these three molecules in patients with OSCC were higher compared to those in healthy controls. Moreover, plasma levels of these three molecules in OSCC patients were significantly correlated with each other and were associated with more-advanced clinical stages and/or a larger tumor size.

Elevated levels of LCN2 expression were detected in several kinds of human tumors (Bauer et al., 2008, Friedl et al., 1999, Furutani et al., 1998, Laurell et al., 2006, Stoesz et al., 1998). Lim et al. reported that tissue expression of LCN2 in ovarian tumors changes with the disease grade, and this is also reflected in serum levels (Lim et al., 2007). The role of LCN2 was also investigated in esophageal squamous cell carcinoma (ESCC), and it was reported that its tissue expression was significantly higher in ESCC than in normal mucosa, and was positively correlated with cell differentiation (Zhang et al., 2007). Recently, it was also demonstrated that the expression of LCN2 in OSCC tissue was significantly correlated with the cancer-cell differentiation status and morphological patterns (epithelial-mesenchymal transition, EMT) (Hiromoto et al., 2011).

It was reported that LCN2 can prevent MMP-9 degradation, increasing its activity by binding and forming the LCN2/MMP-9 complex. Tumor cells excrete elevated levels of LCN2 resulting in an increase in the local concentration of MMP-9, which can affect various aspects of tumor progression (Yan et al., 2001). Moreover, it was reported that tumor cell-secreted MMP-9 promotes angiogenesis *in vivo* in a breast-cancer model where the vascular endothelial growth factor (VEGF)/VEGF

receptor 2 association was shown to be dependent on MMP-9 activity (Mira et al., 2004). In oral cancer, previous reports showed a good correlation between increased tumor vascularity and tumor progression in OSCC (Alcalde et al., 1995, Macluskey et al., 2000, Williams et al., 1994). Previous reports also indicated that VEGF is a vital regulator of OSCC tumor angiogenesis, as evidenced by the high expression level of VEGF in oral cancerous tissues and several OSCC cell lines (Shintani et al., 2004) and the angiogenic inhibition potential of the soluble form of the VEGF 2 receptor *in vivo* (Okada et al., 2010). In this study, we found a significant correlation between plasma levels of LCN2 and LCN2/MMP-9 in patients with OSCC. We further found that plasma levels of LCN2 and the LCN2/MMP-9 complex in OSCC patients were significantly correlated with a larger tumor size. We propose that regulation of OSCC tumor growth by LCN2 might be through increased MMP-9 activity which further promotes VEGF-mediated angiogenic regulation. However, in this study, we did not determine if the higher expression of LCN2 was associated with the often highly vascular OSCC specimens or the role of LCN2 in tumor angiogenesis of OSCC. In our future work, we will collect plasma and tumor tissues from OSCC patients, and further validation studies with LCN2 and the endothelial cell marker, CD 31, antibodies should be conducted to characterize the role of LCN2 in angiogenesis using a larger cohort of OSCC specimens.

In addition to tumor growth regulation, recent evidence suggests that LCN2 expression is associated with the progression of cancer invasion. For example, it was reported that LCN2 serum levels were significant higher in patients with invasive breast cancer while moderate in patients with ductal carcinoma in situ, atypical ductal hyperplasia, and sclerosing adenosis compared to healthy controls (Provatopoulou et al., 2009). Moreover, knockdown of LCN2 in cholangiocarcinoma cells was reported

to suppress their invasive ability through reducing LCN2/MMP-9 complex formation (Nuntagowat et al., 2010). Overexpression of LCN2 can promote the invasive ability of colon carcinoma cells through activating Rac1 signaling (Hu et al., 2009). In contrast to promoting invasion, LCN2 was also reported to inhibit adhesion and invasion in pancreatic cancer, suggesting that LCN2 may be a suppressor of pancreatic cancer progression (Tong et al., 2008). Limitations of this study are a small sample size, and the age and gender not being well matched between the controls and cases. In a future study, we will increase of specimen number and take more OSCC risk factors into account in the analysis to see if we can precisely validate these findings. Additionally, the functional role of LCN2 in OSCC invasion is worth further investigation in our future work.

Improved non-operative diagnostic techniques enable the detection of oral cancer at an earlier stage. At the same time, serum or plasma measurements represent a non-invasive, easily accessible method for the study of biomarkers as screening tools for risk assessment, diagnosis, and prognosis of oral cancer. The present study was designed in an effort to reveal specific biomarkers associated with OSCC progression. Our data suggest that plasma LCN2 and the LCN2/MMP-9 complex are positively correlated with OSCC progression, with potential clinical utility as biomarkers of the OSCC status.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Author contributions

CW Lin and MH Chien designed the study and prepared the manuscript. SF Yang, SW Tseng and CH Lin analyzed the data. YS Hsieh, CP Ko, LH Wei, CW Lin, and MH Chien performed the study plan and collected tissues.

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Table 1. Demographic characteristics and clinical features of oral squamous cell carcinoma (OSCC) patients and healthy controls

Variables	OSCC patients n=195	Controls n=81	p value
Age (years)	53.56 ± 11.43 [a]	51.16 ± 11.52	0.114
Gender: male (%)	189 (99.0%)	77 (95.1%)	0.102
Betel nuts chewing			
Yes	151 (77.4%)	11 (13.6%)	<0.001
No	44 (22.6%)	70 (86.4%)	
Smoking status			
Yes	158 (81.0%)	37 (45.7%)	<0.001
No	37 (19.0%)	44 (54.3%)	
LCN2 (ng/mL)	51.12 ± 2.37	34.88 ± 3.99	0.001 [b]
MMP-9 (ng/mL)	122.63 ± 5.19	68.03 ± 8.80	<0.001 [b]
LCN2/MMP-9 complex (ng/mL)	45.17 ± 2.72	32.68 ± 4.60	0.032 [b]
Tumor T status			
T1	58 (29.7%)	-	
T2	61 (31.3%)	-	
T3	26 (13.3%)	-	
T4	50 (25.6%)	-	
Lymph node status			
N0	137 (70.3%)	-	
N1	30 (15.4%)	-	
N2	27 (13.8%)	-	
N3	1 (0.5%)	-	
Metastasis			
M0	193 (99.0%)	-	
M1	2 (1.0%)	-	
Cell differentiation			
Well differentiated	23 (11.8%)	-	
Moderately or poorly differentiated	172 (88.2%)	-	
Stage			
I	52 (26.7%)		
II	42 (21.5%)		
III	33 (16.9%)		
IV	68 (34.9%)		

[a] Mean ± standard deviation.

[b] After adjusting for betel nut chewing and smoking habits.

Table 2. Correlations of lipocalin (LCN)2, matrix metalloproteinase (MMP)-9, and the LCN2/MMP-9 complex plasma levels with clinicopathological parameters in 195 oral squamous cell carcinoma (OSCC) patients

Variables	LCN2 (ng/mL)	*p* value	MMP-9 (ng/mL)	*p* value	LCN2/MMP-9 complex (ng/mL)	*p* value
	n=195		*n*=195		*n*=195	
Age (years)						
<52.0 (*n*=90)	45.47 ± 31.91	0.036	126.66 ± 81.90	0.523	49.69 ± 51.48	0.191
≥52.0 (*n*=105)	56.01 ± 36.98		119.69 ± 70.10		42.13 ± 26.75	
Gender						
male (*n*=192)	28.57 ± 10.71	0.262	124.43 ± 56.54	0.972	46.75 ± 5.89	0.961
Female (*n*=3)	51.50 ± 35.18		122.88 ± 76.03		45.60 ± 40.48	
Smoking status						
No (*n*=37)	46.26 ± 27.70	0.348	118.58 ± 76.91	0.700	42.93 ± 36.70	0.652
Yes (*n*=158)	52.29 ± 36.53		123.92 ± 75.57		46.25 ± 41.03	
Betel nut chewing						
No (*n*=44)	50.04 ± 31.89	0.813	124.23 ± 64.93	0.895	43.59 ± 23.89	0.705
Yes (*n*=151)	51.47 ± 36.00		122.52 ± 78.70		46.21 ± 43.84	
Tumor T status						
T1+T2 (*n*=119)	45.93 ± 28.19	0.009*	115.33 ± 66.42	0.080	40.75 ± 24.49	0.034*
T3+T4 (*n*=76)	59.30 ± 42.61		134.78 ± 87.36		53.24 ± 55.98	
Lymph node status						
N0 (*n*=137)	49.70 ± 35.47	0.376	116.80 ± 67.44	0.083	43.48 ± 31.02	0.255
N1+N2+N3 (*n*=58)	54.57 ± 34.06		137.34 ± 91.25		50.67 ± 56.21	
Metastasis						
M0 (*n*=193)	50.87 ± 34.79	0.275	122.25 ± 75.25	0.236	45.32 ± 40.22	0.311
M1 (*n*=2)	78.13 ± 65.81		186.17 ± 123.18		74.33 ± 29.69	
Stage						
I+II (*n*=94)	45.68 ± 27.48	0.035*	111.46 ± 64.46	0.041*	40.23 ± 24.27	0.071
III+IV (*n*=101)	56.23 ± 40.32		133.57 ± 86.68		50.62 ± 50.30	
Cell differentiation						
Well differentiated (*n*=23)	46.01 ± 27.29	0.456	115.07 ± 70.90	0.598	41.64 ± 27.69	0.615
Moderately or poorly differentiated (*n*=172)	51.83 ± 35.96		123.96 ± 76.41		46.15 ± 41.59	

* $p<0.05$.

Figure legends

Figure 1. Correlations among plasma lipocalin (LCN)2, matrix metalloproteinase (MMP)-9 and the LCN2/MMP-9 complex expressions in 195 oral squamous cell carcinoma (OSCC) patients. (A) There was a significant correlation between plasma LCN2 and the LCN2/MMP-9 complex (Spearman correlation coefficients $\gamma=0.362$, $p<0.0001$) in the 195 OSCC patients. (B) There was a significant correlation between plasma MMP-9 and the LCN2/MMP-9 complex (Spearman correlation coefficients $\gamma=0.717$, $p<0.0001$) in the 195 OSCC patients. (C) There is a significant correlation between plasma LCN2 and MMP-9 levels (Spearman correlation coefficients $\gamma=0.223$, $p=0.0018$) in the 195 OSCC patients.

Figure 2. Comparison of plasma lipocalin (LCN)2 expression with various tumor sizes and at various stages in 195 OSCC patients. (A) LCN2 levels were compared according to the tumor size and results showed that LCN2 levels at T4 significantly differed between those at T1 and T2. (B) LCN2 levels were compared according to the stage, and results showed that LCN2 levels in stage IV significantly differed from those at stages I and II.

Figure 1A

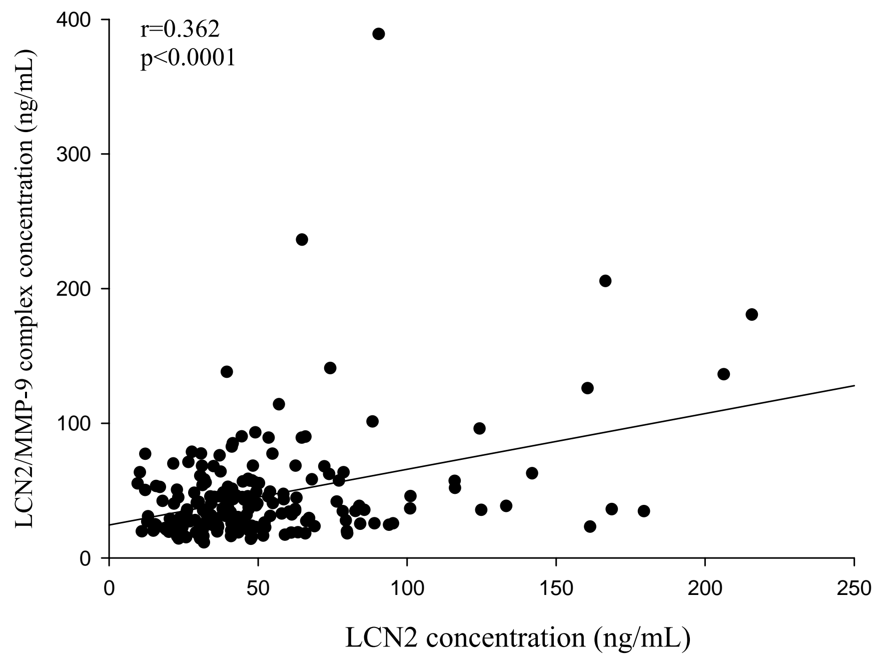


Figure 1B

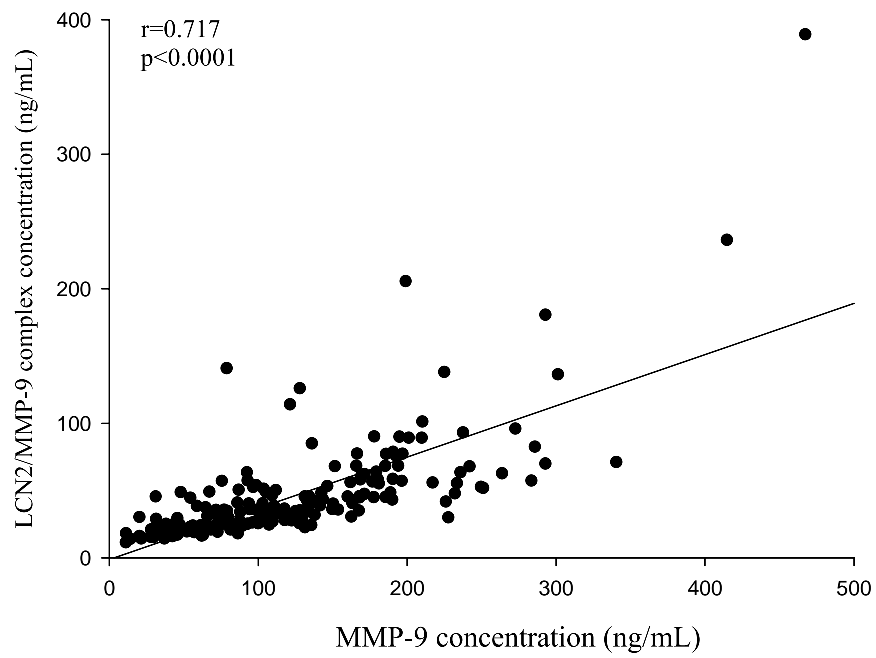


Figure 1C

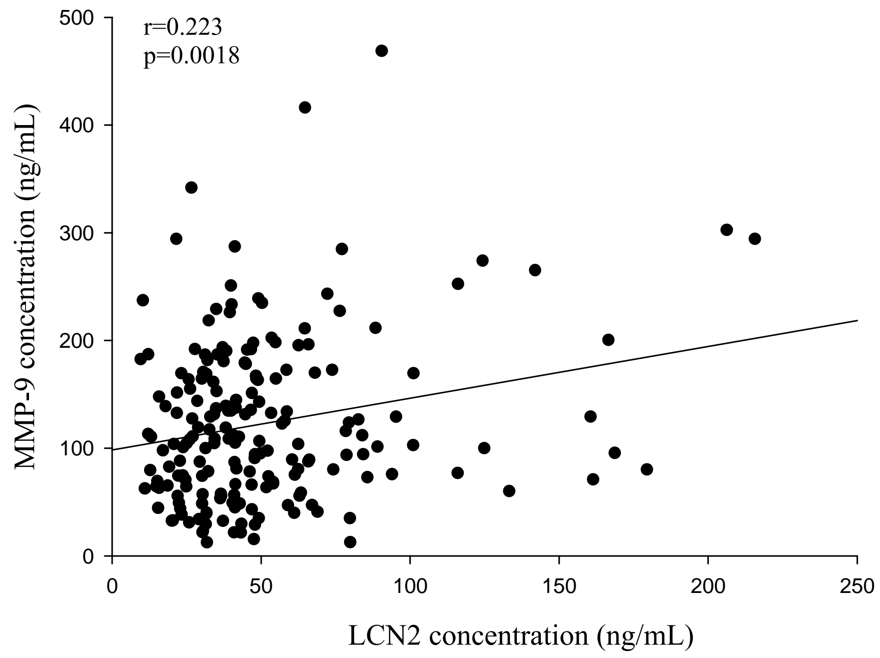


Figure 2A

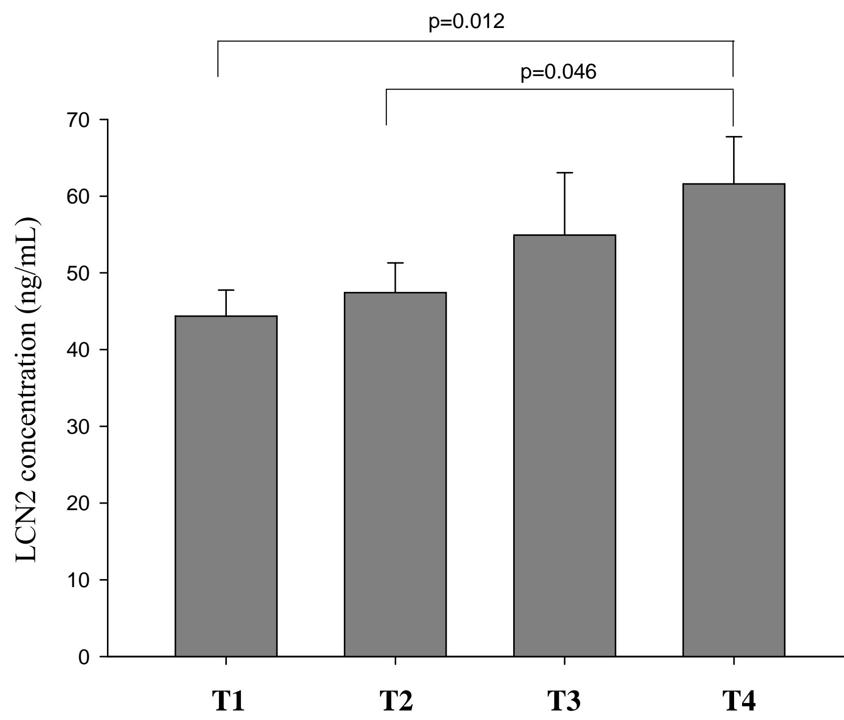
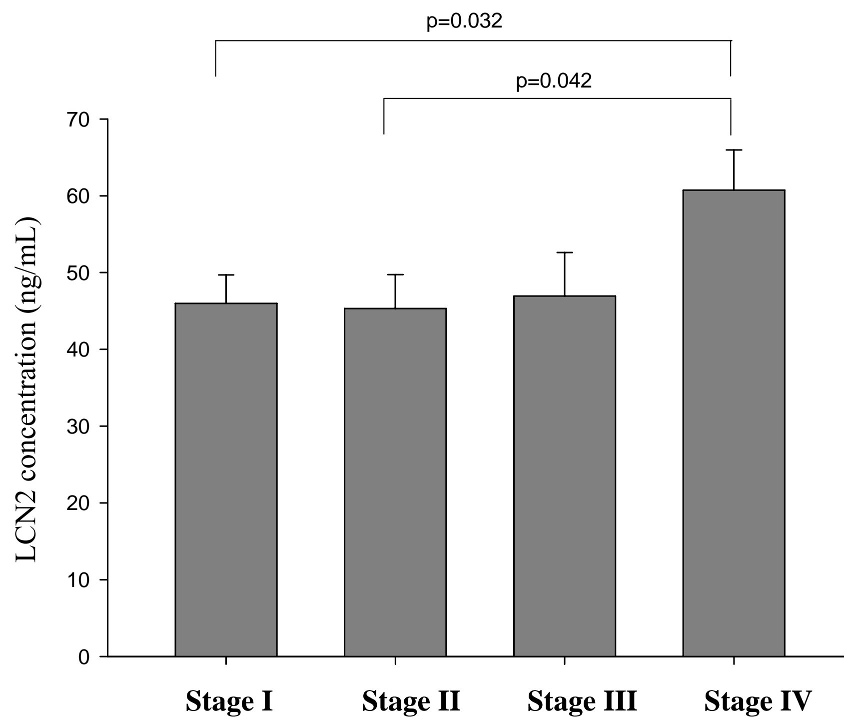


Figure 2B



國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/29

國科會補助計畫	計畫名稱: Lipocalin-2及其MMP-9複合體在口腔黏膜下纖維化及口腔癌的表現及其機制探討
	計畫主持人: 楊順發
	計畫編號: 100-2314-B-040-004- 學門領域: 牙醫學

無研發成果推廣資料

100 年度專題研究計畫研究成果彙整表

計畫主持人：楊順發	計畫編號：100-2314-B-040-004-

計畫名稱：Lipocalin-2 及其 MMP-9 複合體在口腔黏膜下纖維化及口腔癌的表現及其機制探討

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%		
	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	0	0	100%	人次	
		博士生	1	1	100%		
		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	
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		研討會論文	1	1	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	
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		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

<table>
<tr><td>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，請以文字敘述填列。)</td><td>已發 1 篇 SCI 論文</td></tr>
</table>

<table>
<tr><th></th><th>成果項目</th><th>量化</th><th>名稱或內容性質簡述</th></tr>
<tr><td rowspan="8">科教處計畫加填項目</td><td>測驗工具(含質性與量性)</td><td>0</td><td></td></tr>
<tr><td>課程/模組</td><td>0</td><td></td></tr>
<tr><td>電腦及網路系統或工具</td><td>0</td><td></td></tr>
<tr><td>教材</td><td>0</td><td></td></tr>
<tr><td>舉辦之活動/競賽</td><td>0</td><td></td></tr>
<tr><td>研討會/工作坊</td><td>0</td><td></td></tr>
<tr><td>電子報、網站</td><td>0</td><td></td></tr>
<tr><td>計畫成果推廣之參與（閱聽）人數</td><td>0</td><td></td></tr>
</table>

國科會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）

   Lipocalin-2(24p3)是 Lipocalin 蛋白家族的一分子。LCN2 可與基質金屬蛋白水解酶(Matrix metalloproteinase-9；MMP-9)結合而促進 MMP-9 的活性，並且使得 MMP-9 不被降解。 LCN2 除了在人類各種發炎反應會上升，近年來也發現其與腫瘤生成有關。 我們利用酵素聯結免疫吸附法觀察了在口腔鱗狀細胞癌病人(oral squamous cell carcinoma；OSCC)與健康對照組血漿中 LCN2、MMP-9 與 LCN2/MMP-9 複合物的表現。結果顯示 OSCC 病人血漿中 LCN2、MMP-9 與 LCN2/MMP-9 複合物的濃度明顯高於健康對照組(LCN2: $p<0.001$, MMP-9: $p<0.001$, LCN2/MMP-9: $p<0.01$)。並且 LCN2 的表現量與腫瘤大小($p<0.05$)、TNM 分期($p<0.05$)有關，但與淋巴轉移、遠端轉移無關。接著使用 Spearman's Correlation 檢定分析病人血清中 LCN2、MMP-9 與 LCN2/MMP-9 複合物濃度之間的相關性。發現這三者指標兩兩皆呈現正相關。