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Original Article

Decreased expression of CTGF and increased MMP9 are correlated with poor prognosis in nasopharyngeal carcinoma

Xue Li[1*], Xin Xie[1*], Risheng Lei[1], Weiyi Fang[2], Zhen Liu[1], Mao Fang[1]

[1]Department of Pathology, School of Basic Medical Science, Guangzhou Medical University, Guangzhou, P. R. China; [2]Cancer Center, Traditional Chinese Medicine-Integrated Hospital, Southern Medical University, Guangzhou, P. R. China. [*]Equal contributors and co-first authors.

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Abstract: Aims: To investigate the expression correlation of connective tissue growth factor (CTGF) and matrix metalloproteinase 9 (MMP9), and analyze the relationship of their combined expression with clinicopathological characteristics and survival prognosis of NPC patients. Methods: Using real-time PCR, we detected the mRNA expression of CTGF and MMP9 in nasopharyngeal tissues and nasopharyngeal carcinoma (NPC) tissues. Further, we collected the data of CTGF and MMP9 protein expression from NPC samples which had reported in our previous study. The expression correlation of CTGF and MMP9 in mRNA and protein levels was analyzed. Finally, the relationship of the combined expression of CTGF and MMP9 with clinicopathological characteristics and survival prognosis of NPC patients was investigated. Results: MMP9 and CTGF mRNAs were respectively shown to be elevated and decreased in NPC tissues compared to nasopharyngeal tissues. Further, we observed that a negatively correlated tendency was indicated between CTGF with MMP9 in mRNA level (P=0.061). This result was supported by the immunohistochemistry data between CTGF with MMP9 in protein levels (P=0.031). Furthermore, the combined expression model of MMP9 and CTGF proteins was tightly associated with T classification (tumor size) (P<0.001), N classification (lymph node metastasis) (P<0.001), and clinical stage (P<0.001) in NPC patients. Survival analysis indicated that the combined expression of MMP9 and CTGF protein was significantly correlated with the survival prognosis of NPC patients and reduced CTGF and increased MMP9 protein levels showed the poorest survival prognosis than those of other three groups (P<0.001). Finally, the combined expression model of MMP9 and CTGF protein was an independent prognostic factor in NPC patients according to multivariate Cox model analysis (P=0.019). Conclusion: The combination model of up-regulation of MMP9 and down-regulation of CTGF acts as a potential unfavorable prognostic factor for patients with NPC.

Keywords: CTGF, MMP9, nasopharyngeal carcinoma, prognosis

Introduction

Nasopharyngeal carcinoma (NPC), which has a distinctive ethnic and geographic distribution, is one of the most common malignancies with high incidence and mortality rates in Southeast Asia, especially in Hong Kong and Guangdong province, according to the comprehensive analysis of GLOBOCAN and WHO databases [1]. It is reported that genetic susceptibility, Epstein-Barr virus (EBV) infection, dietary and other environmental factors contribute to the development of NPC [2].

CTGF, known as a cysteine-rich, matrix-associated and heparin-binding protein, regulates cell adhesion, migration, proliferation, differentiation, survival, senescence, and apoptosis [3-7] and plays dual roles in various tumors [3, 8]. Recently, we found that reduced CTGF expression was an unfavorable factor promoting NPC growth, migration, and invasion and CTGF might be a significant tumor suppressor participating in the NPC pathogenesis [9].

MMP9, a member of the matrix metalloproteinases (MMPs), plays a critical role in degrading collagen in the extracellular matrix and promotes the metastasis of tumor cells [10, 11]. In previous study, we had also identified overexpressed MMP9 as a poor prognosis factor for the overall survival of NPC patients [12].

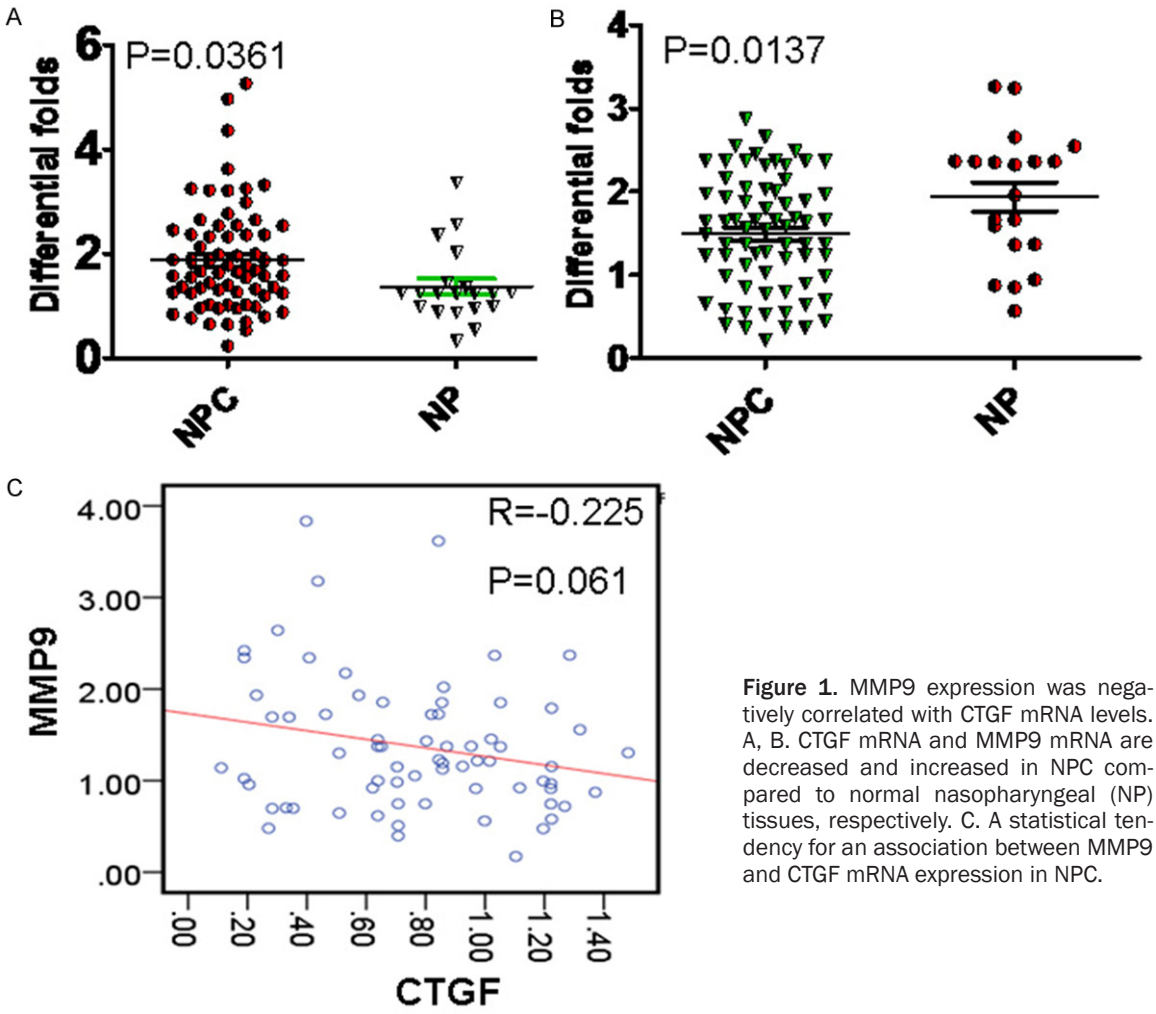

Figure 1. MMP9 expression was negatively correlated with CTGF mRNA levels. A, B. CTGF mRNA and MMP9 mRNA are decreased and increased in NPC compared to normal nasopharyngeal (NP) tissues, respectively. C. A statistical tendency for an association between MMP9 and CTGF mRNA expression in NPC.

However, the relevance of MMP9 and CTGF has not been clarified. In this study, we explored the correlation of CTGF and MMP9 expression, and elucidated their relationships with clinicopathological characteristics and survival prognosis of NPC patients.

Materials and methods

Sample collection

61 fresh NPC samples and 20 fresh non-tumor nasopharyngeal samples were obtained from the People's Hospital of Zhongshan City, China. All cases received no therapy before the diagnosis of NPC. Before the use of these medical materials, we obtained the consent of the patients and the Ethics Committees of this hospital.

Data collection

The data of CTGF and MMP9 protein expression in 142 cases were collected from our previous work [12, 13].

Real-time PCR

RNA was extracted from fresh tissues using Trizol and transcribed into cDNA using reverse transcription reagents (Takara, Shiga, Japan). According to the manufacturer's instructions, PCR reaction was performed on the MX 3000p real-time PCR system (Stratagene, La Jolla, CA, USA) using the SYBR Premix Ex Taq TM II kit (Takara Bio, Inc., Shiga, Japan). The specific primer sets for PCR amplification of CTGF, MMP9 and ARF5 were previously described [12, 13].

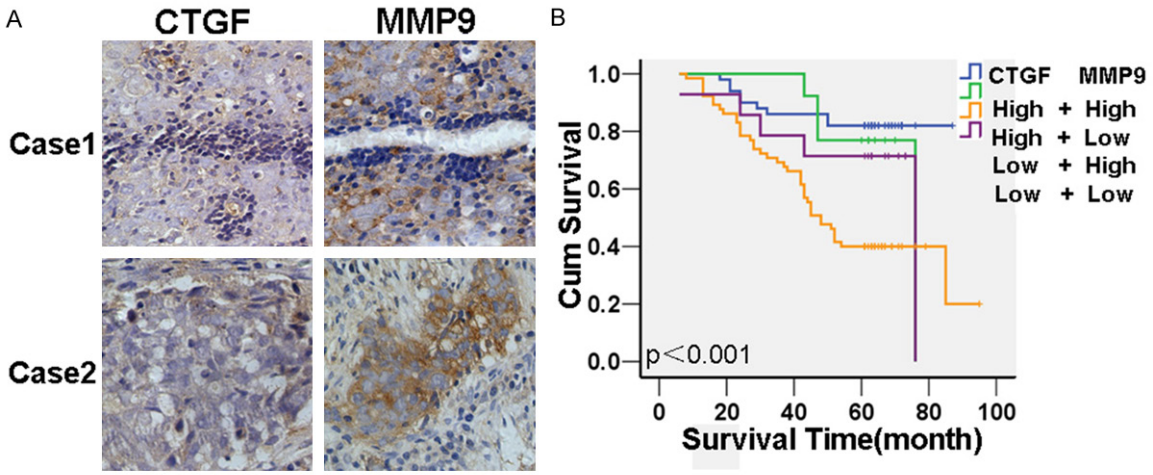

Figure 2. The expression model of CTGF and MMP9 with survival time. A. Representative images showed that CTGF expression was reduced and MMP9 expression was elevated in NPC. B. Low CTGF and high MMP9 expression model was inversely associated with overall survival of NPC patients.

Statistical analysis

All statistical analysis was carried out using SPSS software version 13.0 (SPSS, Inc, Chicago, IL, USA). For comparison of two independent groups, the two-tailed Student's t test was used. Spearman's correlation coefficient was calculated to evaluate the correlation between MMP9 and CTGF expression levels in NPC. The relationship of MMP9 and CTGF expression pattern with clinicopathologic characteristics were analyzed with the χ^2 test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Multivariate Cox proportional hazards model was applied to evaluate the significance of various variables in survival. *P* values of less than 0.05 were considered statistically significant.

Results

MMP9 expression was negatively correlated with CTGF mRNA levels in NPC

Compared with normal NP tissues, elevated MMP9 mRNA levels in NPC were detected by real-time PCR (P=0.0137). However, the expression of CTGF mRNA was significantly lower in NPC than that in normal nasopharyngeal tissues (P=0.0361). The correlation analysis exhibited that there was a statistical tendency for an association between MMP9 and CTGF mRNA expression in NPC (P=0.061) (**Figure 1**).

MMP9 expression was negatively correlated with CTGF protein levels in NPC

The protein expression of MMP9 and CTGF were measured by immunohistochemical staining. It was found that MMP9 staining was strong in NPC specimens with weak expression of CTGF (**Figure 2A**). Furthermore, the assessment of MMP9 and CTGF protein expression levels revealed that MMP9 was negatively relevant to CTGF (P=0.031) (**Table 1**).

Relationship between CTGF/MMP9 expression model and clinicopathological characteristics in NPC patients

Results of correlation of CTGF/MMP9 expression model and clinicopathological characteristics in NPC patients were listed in **Table 2**. We discovered that low CTGF expression and high MMP9 expression was positively associated with T classification (T_1-T_2 versus T_3-T_4) (P< 0.001), N classification (N_0-N_1 versus N_2-N_3) (P<0.001), and clinical stage (I-II versus III-IV) (P<0.001) in NPC. However, the distribution of gender, age, smoking, distant metastasis showed no significant differences between the four CTGF/MMP9 expression pattern (namely,

Table 1. Correlation analysis of CTGF and MMP9 protein expression in NPC

Gene	MMP9			
	Expression Level	High	Low	p
CTGF	High	46	17	P=0.031
	Low	69	10	

Spearman: Correlation conefficient: -0.181.

Table 2. Correlation between the expression of MM9/CTGF and clinicopathological parameters in NPC patients (chi-square test)

Characteristics	n	Expression mode of MMP9/CTGF				P
		LL	LH	HH	HL	
Gender						
Male	91	8	45	7	31	
Female	51	6	20	6	19	0.634
Age (y)						
≥50	68	5	35	5	23	
<50	74	9	30	8	27	0.517
Smoking						
Yes	25	2	12	2	9	
No	117	12	53	11	41	0.980
T classification						
T_1-T_2	96	12	31	10	43	
T_3-T_4	46	2	34	3	7	<0.001
N classification						
N_0-N_1	73	11	15	12	31	
N_2-N_3	69	3	50	1	19	<0.001
Distant metastasis						
Yes	6	1	5	0	0	
No	136	13	60	13	50	0.174
Clinical stage						
I~II	51	11	6	10	24	
III~IV	91	3	59	3	26	<0.001

low CTGF expression and low MMP9 expression, low CTGF expression and high MMP9 expression, high CTGF expression and high MMP9 expression, and high CTGF expression and low MMP9 expression) (P>0.05).

CTGF and MMP9 expression were associated with overall survival time of NPC patients

The connection of CTGF/MMP9 expression model and patients survival was assessed via Kaplan-Meier analysis with the log-rank test to determinate the prognostic value of CTGF and MMP9 expression in NPC. The expression model of CTGF/MMP9 was significantly correlated with the survival of NPC patients, especially, NPC patients with attenuated CTGF expression and increased MMP9 expression had worse survival than those without (P<0.001) (**Figure 2B**). Subsequently, the association between CTGF/MMP9 expression model and prognosis of NPC patients was analyzed by stratifying for T classification, N classification and clinical stage. As shown in **Figure 3**, stratification analysis indicated that reduced CTGF expression and increased MMP9 expression were inversely correlated with survival time for NPC patients with T_3-T_4 tumors (P<0.001) and clinical stage III-IV (P=0.005). Patients with N_0-N_1 and N_2-N_3 stage tumors presented a similar tendency, although the correlation was not statistically significant (P=0.082, P=0.103, respectively), while the prognosis of patients with T_1-T_2 stage tumors and clinical stage I-II was not associated with the CTGF/MMP9 expression pattern (P>0.05).

CTGF/MMP9 expression model was an independent prognostic factor for NPC patients

Univariate analysis prompted that the overall survival of NPC patients were significantly associated with clinicopathological variables, including smoking (P=0.032), T classification (P< 0.001), N classification (P<0.001), M classification (P<0.001), clinical stage (P<0.001), and CTGF/MMP9 expression pattern (P<0.001). Moreover, Multivariate analysis was used to elucidate whether the MMP9/CTGF expression model was the independent prognostic factor for NPC. Intriguingly, the results illustrated that chemotherapy (P=0.031), T classification (P= 0.011), M classification (P=0.008), and MMP9/CTGF expression model (P=0.019) were independent poor prognostic indicators in NPC patients (**Table 3**).

Discussion

Nasopharyngeal carcinoma, a head and neck epithelial malignancy, occurs frequently in Southern China [14]. Patients with NPC that are typically diagnosed in advanced stages have a poor prognosis because of late presentation of lesions, limited knowledge of molecular pathogenesis, lack of early detection biomarkers, and poor response to available therapies [15]. In the present study, we demonstrated that low

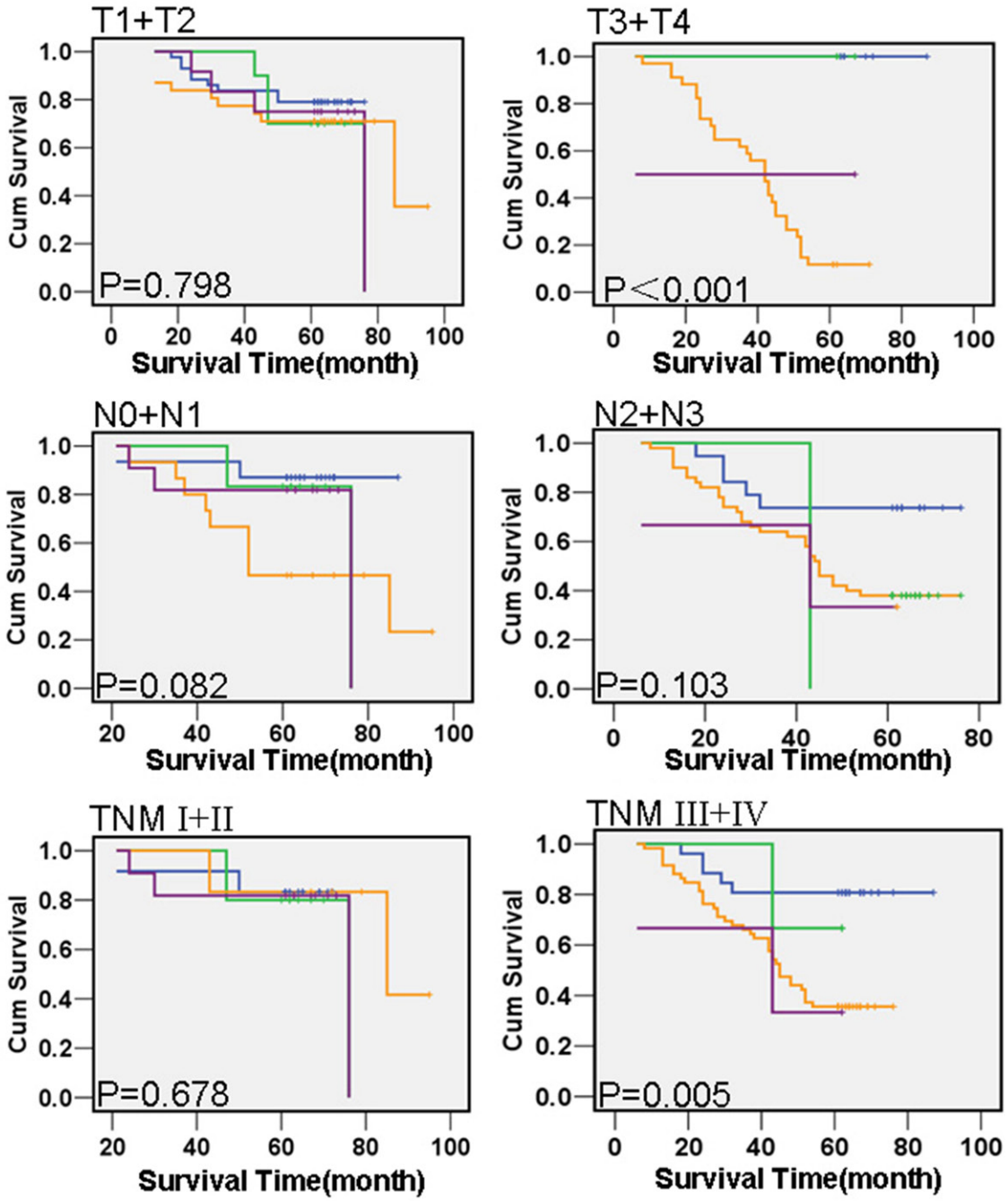

Figure 3. The relationship of CTGF and MMP9 expression model with prognosis of NPC was assessed by stratification analysis. The down-regulation of CTGF and up-regulation of MMP9 were inversely correlated with survival time for NPC patients with T_3-T_4 tumors and clinical stage.

CTGF combined with high MMP9 expression which can serve as a potential biomarker for the clinical prognosis or diagnosis of NPC and anti-cancer strategies targeting the two genes may be appropriate for the treatment of Nasopharyngeal carcinoma.

We have recently documented that CTGF controls the expression of MMP9 in NPC [9], and inhibition of CTGF induces acceleration of MMP9 expression. In the present work, we further proved that CTGF was negatively associated with MMP9 expression. In contrast, a study

Table 3. Summary of univariate and multivariate Cox regression analysis of overall survival duration

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age						
≥50 vs. <50 years	0.087	1.582	0.936-2.676	0.483	1.218	0.702-2.114
Gender						
Male vs. female	0.116	1.643	0.885-3.050	0.891	1.049	0.529-2.080
Smoking						
Yes vs. No	0.032	1.915	1.057-3.469	0.160	1.617	0.828-3.159
Biotherapy						
Yes vs. No	0.667	0.734	0.179-3.011	0.313	2.211	0.474-10.315
Chemotherapy						
Yes vs. No	0.066	1.635	0.967-2.762	0.031	1.906	1.059-3.430
Radiotherapy						
Yes vs. No	0.322	0.719	0.374-1.382	0.418	0.745	0.366-1.518
T classification						
T_1-T_2 vs. T_3-T_4	0.000	3.160	1.878-5.317	0.011	2.369	1.215-4.619
N classification						
N_0-N_1 vs. N_2-N_3	0.000	2.806	1.600-4.922	0.127	2.034	0.818-5.058
M classification						
M_0 vs. M_1	0.000	12.110	4.945-29.660	0.008	3.879	1.431-10.510
Clinical stage						
I-II vs. III-IV	0.001	2.930	1.543-5.563	0.938	1.046	0.337-3.248
MMP/CTGF expression						
LL vs. LH vs. HL vs. HH	0.001	0.641	0.495-0.828	0.019	0.670	0.480-0.935

conducted by Tank suggested that CTGF silencing markedly reduced the expression of MMP2 and MMP9 in cardiac fibroblasts [16], and another report [17]. Supported the results from Tank. The difference in types of study samples may explain this contradiction. Moreover, NPC patients with reduced expression of CTGF and increased expression of MMP9 had worse prognosis than those without, suggesting that the expression pattern of CTGF and MMP9 is a clinically significant biomarker for NPC prognosis. The stratification analysis disclosed that low expression of CTGF and high expression of MMP9 were correlated with unfavorable prognosis in NPC patients with T_3-T_4 and clinical stage III-IV cancer, not T_1-T_2 stage and clinical stage I-II cancer. CTGF is involved in various regulatory processes, such as angiogenesis, chondrogenesis, osteogenesis, fibrosis formation, diabetic nephropathy, and tumor development [4] and exerts the distinct effects on different types of cancers [18-26]. Our previous study has verified that the inhibition of CTGF contributes to the overexpression of MMP9. MMP9 is a Zn^{2+} dependent endopeptidase that mediates the degradation of extracellular matrix protein [27]. The degradation process is validated as a key step in tumor invasion and metastasis [28, 29]. Therefore, our results further supported that CTGF and MMP9 is mainly responsible for advanced tumor development. Nevertheless, our research achieved no significant results in patients with N_0-N_1 and N_2-N_3 stage tumors, replication studies with large sample sizes are needed to confirm the outcome.

Finally, we assessed the expression model of CTGF and MMP9 as an independent prognostic factor. Univariate analysis implied that the overall survival rate of patients was strikingly correlated with smoking, T classification, N classification, M classification, clinical stage and MMP9/CTGF expression model. Multivariate analysis further clarified that chemotherapy; T classification, M classification, and MMP9/CTGF expression model were independent prognostic indicators. Overall survival of NPC patients is correlated inversely with classification of T and M, clinical stage and MMP9/CTGF

expression pattern, and positively proportional to chemotherapy. Our work emphasized that the CTGF and MMP9 expression can be a potential biomarker in NPC.

Conclusion

Taken together, this study documented that there was a negative association between MMP9 and CTGF expression and their expression level was notably related with clinical characteristics and prognosis of NPC patients. Additionally, elevated MMP9 and reduced CTGF expression model was identified as an independent prognostic indicator and unfavorable biomarker of prognosis in NPC patients. Our research may provide novel therapeutic strategies for NPC. However, because of limited dataset, further research is still needed to verify these findings.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Mao Fang and Zhen Liu, Department of Pathology, School of Basic Medical Science, Guangzhou Medical University, Xinzao, Panyu District, Guangzhou 511436, P. R. China. Tel: 86-37103206; Fax: 86-37103206; E-mail: fangmao82@126.com (MF); narcissus_jane@163.com (ZL)

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