

Expression of matrix metalloproteinase-9 in nasopharyngeal carcinoma and association with Epstein-Barr virus infection

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Abstract: Objective: To evaluate the expression of matrix metalloproteinase-9 (MMP9) in nasopharyngeal carcinoma and the association between MMP9 and Epstein-Barr virus infection. Methods: The MMP9 expression was studied by immunohistochemical analysis; and Epstein-Barr virus encoded small nuclear mRNA-1 (EBER-1) produced by in situ hybridization were examined in 41 nasopharyngeal carcinoma sections, and the relation between them, and the associations of MMP9 with clinical features were statistically analyzed. Results: Positive expression rate of MMP9 was 73.17%. The expression of MMP9 showed significant positive correlation with the expression of EBER-1 ($\gamma=0.483$, $P=0.001$). There was significant association of MMP9 expression with lymph nodes metastasis and clinical stage ($P<0.001$), non-significant association with age, gender, pathological classification and T classification. Conclusions: The highly pronounced expression of MMP9 is associated with cervical lymph nodes metastasis. Epstein-Barr virus can enhance NPC metastasis by up-regulating the expression of MMP9.

Key words: Matrix metalloproteinase-9, EB virus, Nasopharyngeal carcinoma

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a highly metastatic and invasive malignant tumor and is more metastatic than any other head and neck carcinomas. Approximately 90% of patients show cervical lymph nodes metastasis as the most frequently found nasopharyngeal carcinoma.

Epstein-Barr virus (EBV), a ubiquitous human gamma herpes virus, is associated with Hodgkin disease and several malignant tumors such as endemic Burkitt's lymphoma, stomach carcinoma and NPC. The consistent association of EBV with NPC was established by the following findings: the presence of EBV DNA, RNA and proteins in the tissues of NPC; evidence that the tumor cells are composed of single EBV clone infected cell; and

increased antibodies against EBV antigens in NPC patients. Among the methods for detecting EBV infection in NPC tissues, the most sensitive one is in situ hybridization with EB virus encoded small nuclear mRNA-1 (EBER-1) with a very high positive rate, even 100%.

The interstitial stroma of tissues does not normally contain preexisting passageway for cells. The basal membrane (BM) is an insoluble continuous but flexible structure impermeable to large proteins. This type of extracellular matrix (ECM) becomes focally permeable to cell movement only during tissue healing and remodeling, inflammation and neoplasia. Cell infiltration of the EMC undoubtedly depends on multiple factors including properties of both the infiltrating cells and associated host cells as well as properties of the matrix

itself. Although metastasis is a cascade of linked sequential steps involving host-tumor interaction, ECM disruption is a crucial step in which neoplastic cells leave the primary lesion and invade local and distant host tissues. Liotta (1986a; 1986b; 1992) proposed a three-step hypothesis describing the sequence of biochemical events during tumor cell invasion of the EMC. The first step is tumor cell attachment via cell surface receptors that specially bind to the matrix components such as lamin (for BM) and fibronectin (for the stroma). The anchored tumor cell next secretes hydrolytic enzymes (or induces host cells to secrete enzymes) which can locally degrade the matrix (including degradation of the attachment components). Matrix lysis most probably occurs in a highly localized region close to the tumor cell surface. The third step is tumor cell migration to the matrix region modified by proteolysis. Continued invasion of the matrix may take place by cyclic repetition of these three steps.

Enzymes which can effectively degrade ECM include metalloproteinases (MMPs), heparanases, serine- and thiol-dependent proteinases, and so on, but the most representative one is MMPs, because their active substrates are the most important components of materials which maintain cellular structure and matrix.

More than 20 kinds of MMPs (a gene family of zinc-containing endopeptidases) have been found since the first MMP was discovered in 1962. Among MMP families, MMP9 (the 92-kD type IV collagenase/gelatinase B) which selectively degrades type IV collagen (a major component of EMC) is reported to be markedly associated with tissue invasion and metastasis. Many reports have been published about the association of MMP9 with the metastasis of a large variety of cancers such as those of lung, prostate, breast, colon, head, and neck; MMPs in NPC have rarely been reported.

We are very interested in whether there is probable relation or consistent association between MMP9 and NPC metastasis; and if there is any correlation between MMP9 and EBV infection. So we investigated the expression of MMP9 by immunohistochemical analysis and EBER-1 by in situ

hybridization in 41 specimens of NPC in patients and studied the relation between them. In addition, we assessed the correlation of MMP9 expression with clinical features. We want to confirm our hypothesis that MMP9 is associated with EBV infection and the lymph nodes metastasis of NPC.

MATERIAL AND METHODS

Source of tissue samples

The experimental group consisted of 41 nasopharyngeal biopsy specimens from NPC patients who underwent treatment in Sir Run Run Shaw Hospital from 1998 to 2002, with 1 well-differentiated squamous cell carcinoma, 11 moderately-differentiated squamous cell carcinomas, 16 weakly-differentiated squamous cell carcinomas and 13 undifferentiated carcinomas. They included 26 male patients and 9 female patients. More details can be seen in Table 1. The control group consisted of ten nasopharyngeal biopsy specimens of chronic pharyngitis from 7 male patients and 3 female patients. All the tissue samples were fixed in 10% formaldehyde solution after being obtained, then embedded in paraffin. Five micrometer thick sections were prepared for examining the expression of MMP9 and EBER-1.

Immunohistochemical analysis and in situ hybridization

Standard Streptavidin-peroxidase immunohistochemical procedure was performed at room temperature unless otherwise stated. Briefly, after being dewaxed and rehydrated, all the sections were antigen-repaired by microwave heating in citromalic acid buffer (96 °C, 20 minutes). Endblocker (Zymed Corp., U.S.A.) was used to perform blocking for 10 minutes. Then the sections were mounted with 10% normal sheep serum (MaiXin Corp., China) for 10 minutes. After that, all the sections were incubated with mouse-anti-human MMP9 monoclonal antibody (Zymed Corp., U.S.A.) at 4 °C overnight and then they were exposed to a universal secondary antibody (Zymed Corp., U.S.A.) for 30 minutes, followed by incubation

Table 1 Clinicopathologic features and expression score of MMP9 and EBER-1

Case no.	Gender	Age (yrs)	Histology	TNM	Stage	MMP9 (%)	EBER-1 (%)
1	f	32	m-diff	T ₂ N ₁ M ₀	III	93.2	77.5
2	m	63	w-diff	T ₂ N ₀ M ₀	II	0.0	72.5
3	m	61	undiff	T ₃ N ₂ M ₀	IV	80.1	85.3
4	f	59	m-diff	T ₄ N ₁ M ₀	IV	74.6	86.9
5	m	69	undiff	T ₂ N ₀ M ₀	II	2.7	60.5
6	m	57	m-diff	T ₂ N ₃ M ₀	IV	55.6	70.9
7	m	52	undiff	T ₂ N ₂ M ₀	IV	53.9	59.7
8	m	49	undiff	T ₁ N ₂ M ₀	III	35.1	65.5
9	m	62	m-diff	T ₂ N ₃ M ₀	IV	100.0	100.0
10	f	73	w-diff	T ₂ N ₂ M ₀	IV	62.8	79.1
11	f	60	w-diff	T ₁ N ₁ M ₀	III	1.5	65.1
12	f	49	w-diff	T ₂ N ₃ M ₀	IV	39.4	71.5
13	m	47	w-diff	T ₂ N ₃ M ₀	IV	72.1	77.6
14	m	62	undiff	T ₂ N ₀ M ₀	II	3.2	75.0
15	f	62	w-diff	T ₄ N ₂ M ₀	IV	79.2	77.6
16	m	59	w-diff	T ₃ N ₂ M ₀	IV	58.1	63.3
17	m	54	w-diff	T ₁ N ₂ M ₀	IV	100.0	100.0
18	f	29	undiff	T ₂ N ₂ M ₀	IV	75.4	90.0
19	m	38	w-diff	T ₂ N ₀ M ₀	II	4.6	87.2
20	f	43	m-diff	T ₄ N ₀ M ₀	IV	4.0	58.9
21	m	65	undiff	T ₁ N ₃ M ₀	IV	46.1	83.4
22	m	67	w-diff	T ₃ N ₀ M ₀	III	40.2	68.2
23	m	43	undiff	T ₂ N ₀ M ₀	II	2.7	4.8
24	m	48	w-diff	T ₃ N ₂ M ₀	IV	36.5	63.4
25	f	47	m-diff	T ₄ N ₀ M ₀	IV	19.0	50.6
26	m	54	m-diff	T ₃ N ₁ M ₀	III	66.1	80.6
27	f	57	m-diff	T ₂ N ₁ M ₀	III	5.0	78.6
28	m	61	m-diff	T ₄ N ₂ M ₀	IV	80.1	54.4
29	m	49	undiff	T ₃ N ₂ M ₀	IV	51.8	80.8
30	m	62	undiff	T ₃ N ₁ M ₀	III	31.8	75.1
31	m	55	undiff	T ₁ N ₁ M ₀	III	49.6	41.1
32	m	50	undiff	T ₂ N ₂ M ₀	IV	78.6	52.1
33	m	44	w-diff	T ₃ N ₁ M ₀	III	87.5	68.4
34	m	65	w-diff	T ₂ N ₁ M ₀	III	0.0	63.7
35	f	54	m-diff	T ₁ N ₀ M ₀	I	1.6	69.8
36	m	74	w-diff	T ₂ N ₁ M ₀	III	1.9	29.2
37	f	52	w-diff	T ₁ N ₃ M ₀	IV	85.3	95.0
38	m	61	m-diff	T ₂ N ₂ M ₀	IV	48.9	73.6
39	f	37	undiff	T ₂ N ₂ M ₀	IV	97.1	100.0
40	f	63	w-diff	T ₂ N ₂ M ₀	IV	23.4	53.4
41	f	53	wl-diff	T ₂ N ₁ M ₀	III	50.3	38.6

m: male; f: female; wl-diff: well differentiated; m-diff: moderately differentiated; w-diff: weakly-differentiated; undiff: undifferentiated

with horseradish peroxidase-conjugated streptavidin for 30 minutes. At last, the sections were stained by diaminobenzidine, and hematoxylin as nuclear counterstaining.

EBER-1 in situ hybridization was performed at room temperature according to EBER-1 in situ hybridization kit introduction of Novocastra Corp. Paraffin sections were dewaxed in xylene and hy-

drated through a graded ethanol series (99%, 95%) and water. Then the slides were treated with proteinase K (10 $\mu\text{g}/\text{ml}$, Novocastra Corp., U.S.A.) at 37 °C for 30 minutes, washed in water, dehydrated through ethanol graded series (95%; 99%) and air-dried. After incubation with fluorescein-conjugated EBER-1 probes (Novocastra Corp., U.S.A.) covered with cover glasses at 37 °C for 120 minutes, the slides were washed in Tris-buffered saline (TBS, 50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH=7.6) containing 0.1% Triton X-100, followed by block solution (normal rabbit serum 1:5 diluted into TBS buffer containing 0.1% Triton X-100 and 3% BSA) for 10 minutes. Then the block solution was tipped off and the slides were incubated with rabbit F (ab') anti-FITC/AP (Alkaline phosphatase-conjugated antibody to fluorescein isothiocyanate [affinity-isolated rabbit F(ab')], Novocastra Corp., U.S.A.) diluted 1:150 in TBS containing 0.1% Triton X-100 and 3% BSA for 30 minutes. After being washed with TBS and alkaline phosphatase substrate buffer (100 mmol/L Tris/HCl, 50 mmol/L MgCl_2 , 100 mmol/L NaCl, pH=9.0), alkaline phosphatase activity was initiated by covering sections with Enzyme Substrate [50 \times concentrated 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; BCIP/NBT (Novocastra Corp., U.S.A.) was diluted 1:50 in alkaline phosphatase substrate buffer] and then incubated in the dark overnight. All the sections were counterstained with hematoxylin at last.

In addition, positive and negative control slides were processed in parallel. Previously identified MMP9 positive breast carcinoma slides were used as positive control for MMP9 offered by Zymed Corp. and EBER-1 positive NPC slides for EBER-1 positive control were offered by Novocastra Corp. PBS buffer was (instead of the primary antibody) negative control in immunohistochemical analysis and Control Probe offered by Novocastra Corp. was used in in situ hybridization.

Evaluation of the specimens

All specimens were independently evaluated by two pathologists without knowledge of clinical data and the evaluation of one was compared with

that of the other. The examiners each selected 3 representative fields containing greater than 100 tumor cells and counted the positive stain cells and the total number of tumor cells. The average percentage of positive stain cells was calculated as the MMP9 expression score and EBER-1 expression score. The slide was defined positive stain if its expression score was larger than 5%, otherwise negative. All staining for MMP9 and EBER-1 were repeated at least 2 times in sequential sections to assess reproducibility.

Statistical analysis

All figures were analyzed statistically with SPSS 10.0 software in a personal computer. The Pearson Coefficient was used to analyze the correlation between the expression of MMP9 and EBER-1. The expressions of MMP9 in relation to the clinicopathologic data were analyzed by using T test and analysis of variance.

RESULTS

Stain features of MMP9 and EBER-1

MMP9 predominantly immunolocalized at the cytoplasm and EBER-1 positive stain exclusively localized at nucleus. MMP9 positive cell had a yellow or brown yellow cytoplasm and blue nucleus. EBER-1 positive cell had a bluish purple or dark purple nucleus and no stain at the cytoplasm. MMP9 positive stain also could be clearly observed in interstitial cells and vascular endothelial cells in the stroma surrounding cancer nests. No EBER-1 positive stain was found in the stroma. Except for a few gland cells and vascular endothelial cells, no MMP9 positive stain was observed in other cells in control group sections. EBER-1 positive rate of control group was 0%. Figs.1–10 shows microscopic photos of some sections.

Association of MMP9 with clinicopathologic data

As shown in Table 2, the MMP9 expression score of N_0 group (without lymph nodes metastasis) was $8.67 \pm 13.07\%$ (mean \pm standard deviation [SD])

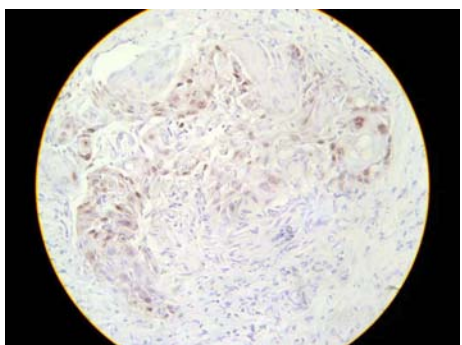


Fig.1 EBER-1 positive stain in well-differentiated NPC (×400)

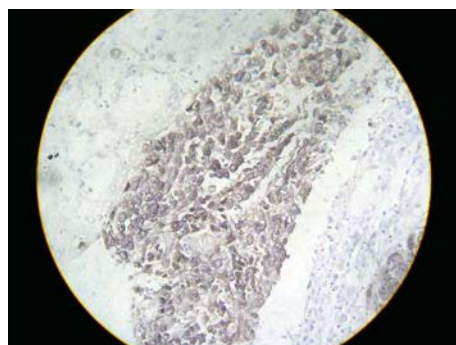


Fig.5 EBER-1 positive stain in weakly-differentiated NPC (×400)

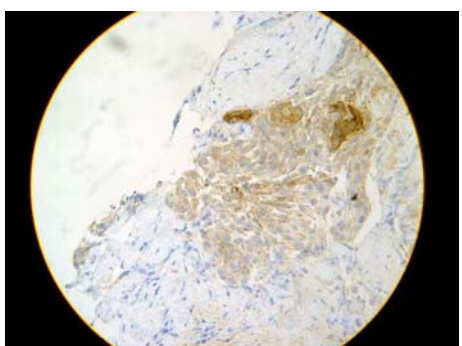


Fig.2 MMP9 positive stain in well-differentiated NPC (×400)

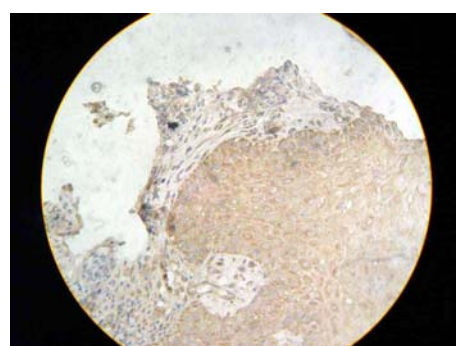


Fig.6 MMP9 positive stain in weakly-differentiated NPC (×400)

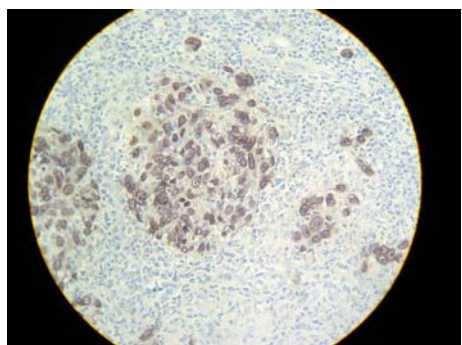


Fig.3 EBER-1 positive stain in moderately-differentiated NPC (×400)

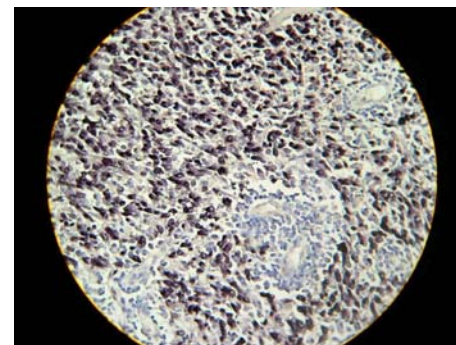


Fig.7 EBER-1 positive stain in undifferentiated NPC (×400)

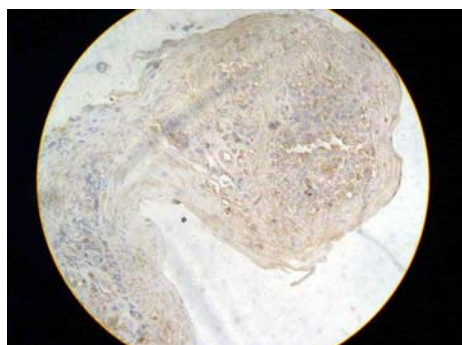


Fig.4 MMP9 positive stain in moderately-differentiated NPC (×400)

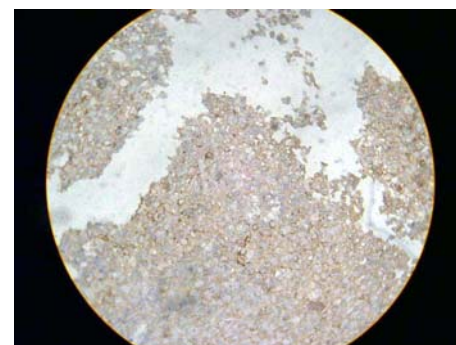


Fig.8 MMP9 positive stain in undifferentiated NPC (×400)

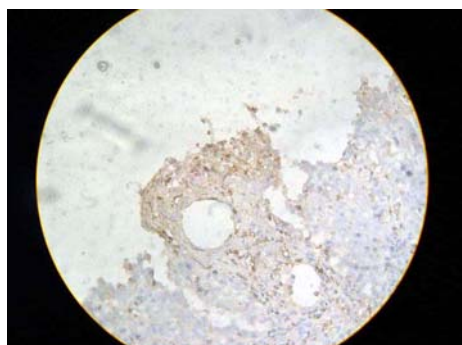


Fig.9 MMP9 positive stain in vascular endothelial cells in the stroma surrounding cancer nests ($\times 400$)

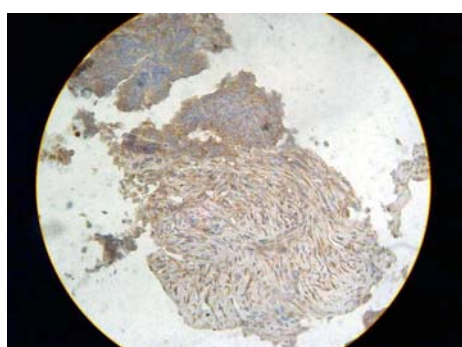


Fig.10 MMP9 positive stain in interstitial cells in the stroma surrounding cancer nests ($\times 400$)

and that of N_{1-3} group (with lymph nodes metastasis) was $56.91 \pm 29.37\%$ (mean \pm SD); Significant difference was observed to exist between them ($P < 0.0001$). MMP9 expression score of early NPC group (I, II stage) was $2.47 \pm 1.55\%$ (mean \pm SD) and that of advanced NPC group (III, IV stage) was $53.83 \pm 30.16\%$ (mean \pm SD); there also was statistically difference ($P < 0.0001$). Thus, as shown in Table 2, MMP9 expression positively correlated with lymph nodes metastasis and clinical stage and did not associate with age (≥ 60 years group, < 60 years group), gender (male group, female group), T classification (T_{1-2} group, T_{3-4} group) and histology classification (moderately-differentiated squamous cell carcinomas group, weakly-differentiated squamous cell carcinomas group, undifferentiated carcinomas group). All the statistic data and the P value are shown in Table 2.

Correlation between MMP9 and EBER-1 expression

The mean MMP9 expression score of the experimental group was $46.32 \pm 33.33\%$ (mean \pm SD)

Table 2 Association of MMP9 expression score and clinicopathologic features

Factor	No.	MMP9 expression score (mean \pm SD)	Statistic	P value
Total No.	41	$46.32\% \pm 33.33\%$		
Gender				
Male	26	$45.66\% \pm 32.43\%$	$t=0.164$	0.871
Female	15	$47.45\% \pm 35.97\%$		
Age (yrs)				
≥ 60	16	$37.62\% \pm 34.76\%$	$t=1.350$	0.185
< 60	25	$51.88\% \pm 31.85\%$		
Histology				
wl-diff	1	50.30% [#]		
m-diff	11	$49.83\% \pm 36.90\%$	$F=0.120$	0.890
w-diff	16	$43.28\% \pm 35.42\%$		
undiff	13	$46.78\% \pm 31.24\%$		
T classification				
T_{1-2}	28	$42.50\% \pm 35.87\%$	$t=1.078$	0.288
T_{3-4}	13	$54.54\% \pm 26.49\%$		
N classification				
N_0	9	$8.67\% \pm 13.07\%$	$t=4.763^*$	< 0.0001
N_{1-3}	32	$56.91\% \pm 29.37\%$		
Stage grouping				
I, II	6	$2.47\% \pm 1.55\%$	$t=4.128^*$	< 0.0001
III, IV	35	$53.83\% \pm 30.16\%$		

[#]This marked datum was deleted during statistical analysis because this group had only one case

* The symbol indicated the datum was statistically significant

and MMP9 positive rate was 73.17% (30/41). The mean EBER-1 expression score was $69.32 \pm 19.19\%$ (mean \pm SD) and its positive rate was 97.56% (40/41). Fig.11 of the distribution of the MMP9 expression score and the EBER-1 expression score of each case shows that the expression of MMP9 significantly correlated with EBER-1 expression ($r=0.483$; $P=0.001$).

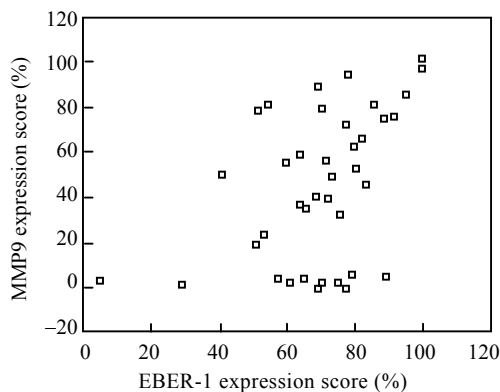


Fig.11 correlation between the scores of MMP9 expression and EBER-1 expression in NPC shows the MMP9 expression has a significant correlation with EBER-1 (the Pearson correlation coefficient; $r=0.483$, $P=0.001$)

DISCUSSION

The current study clearly showed significant positive correlation of MMP9 protein expression with lymph nodes metastasis nasopharyngeal carcinoma; and close association between MMP9 expression and EBV infection. Generally, MMP9 expression is produced not only by tumor cells but also a variety of stroma cells and is predominantly induced by tumor cells. We observed MMP9 positive stain at some interstitial cells (i.e. fibroblasts and endothelial cells) around cancer nests in our sections, which may show that tumor cells could not only secrete MMP9 protein themselves but induce host cells to secrete the enzyme to degrade the EMC and slow down the metastasis of tumor cells. The authors are not aware of reports MMP9 being a target of chemotherapy combined with radiotherapy in NPC. Matrix metalloproteinase inhibitor (Roche 28-2653), with high sensitivity to MMP9,

could reduce tumor growth and prolonged survival in a prostate cancer standard rat model (Lein *et al.*, 2002). So we thought that chemotherapy combined with radiotherapy might be useful for decreasing the metastatic rate and improving survival rate in NPC patients. We also compared our experimental results with other reports. Zhang *et al.*(1999) reported that MMP9 expression was significantly associated with lymph nodes metastasis and histology classification in NPC. There were 10 well-differentiated squamous cell carcinomas and 16 weakly-differentiated squamous cell carcinomas in his experimental group which greatly different from the pathological conformation of our experimental group. Horikawa *et al.*(2000) reported that MMP9 expression was correlated with lymph nodes metastasis in NPC, but had no association with clinical stage and pathology classification, mainly the clinical stage classification criterion we selected (which is generally used in China) was different from theirs. According to all the above findings, we thought that MMP9 was confirmably correlated with cervical lymph nodes metastasis in NPC; although the relation of MMP9 with clinical stage and histology required additional experiments to clarify.

Our study confirming that EBV infection was positively correlated with the expression of MMP9 indicated that EBV plays a very important role in nasopharyngeal carcinoma occurrence and growth, and can adversely influence its metastasis by up-regulating the expression of MMP9 protein. The EBV mechanism that regulates MMP9 is still a mystery to us all. As other MMP families, MMP9 is tightly regulated with multi-stepped mechanism. Briefly, the regulation is on three levels: transcription of MMP9, translation, secretion and activation of MMP9 and regulation after activation. Transcription of MMP9 induces a variety of factors, such as 12-o-tetradecanoyl-phorbol-13-acetate, cytokine (i.e. interleukin 1), oncogenes (H-ras and v-src) and tumor necrosis factor α , and so on. These factors induced MMP9 transcription mediated by activation of cellular transcriptional factors such as NF- κ B, SP-1 and AP-1. EBV gene products probably also contribute to regulation of MMP9 by

regulating these factors or cellular transcriptional factors directly. EBV encoded latent membrane protein-1 (LMP-1) can enhance the MMP9 expression in EBV negative NPC cells by up-regulating the expression of NF- κ B and AP-1 (Takeshita *et al.*, 1999). MMP9 can also be induced by Z protein that was encoded by EBV immediate-early gene BZLF-1 and can up-regulate AP-1 transcription (Yoshizaki *et al.*, 1999). Aside from the regulation of transcription, some researchers reported regulation on other levels. Huang *et al.* (2000) reported that LMP1, LMP2A and EBNA 2 (EB virus nuclear antigen 2) could selectively activate alpha integrin in EBV infect human B cells; previous researches identified that the alpha integrin playing an important role in epithelial-derived cell migration, cell growth and tumor invasion/metastasis. Their experiment also confirmed that alpha antisense oligonucleotides specifically reduced cell surface expression of alpha (ν) integrins, inhibited cell growth in serum, reduced cell invasion in matrigels and decreased expression of MMP9. They did not demonstrate the actual mechanism. Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenic inhibitors of MMPs. The paper's authors are not aware of reports confirming the relation between EBV infection and TIMP in NPC, although Gaudin *et al.* (2000) reported the improved expression of TIMP1 in EBV immortalized B lymphocyte cellular model. Our experiment result showed positive correlation between EBER and MMP9 expression, although additional studies of the molecular mechanism of EBER-mediated MMP9 transcription are required. The above findings show that EBV mechanism regulating MMP9 was multi-level and multi-stepped; and that the regulation of transcription may be relatively more important.

Except for MMP, angiogenesis is also a key step in tumor metastasis. Massive formation of blood vessels at the tumor site increases the opportunity for tumor cells to enter the circulation. Recently, some researches revealed that EBV encoded products also involved in angiogenesis in NPC. Vascular endothelial growth factor (VEGF), the basic fibroblast growth factor (bFGF) and in-

terleukin 8 (IL-8) are prominent angiogenic molecular factors, which had been demonstrated to influence microvessel synthesis in various tumors. EBV encoded LMP-1 can induce IL-8 expression probably contributing in part to angiogenesis in NPC through the NF- κ B binding site, although VEGF, and bFGF expression are insignificantly correlated to LMP-1 expression (Yoshizaki *et al.*, 2001). It was confirmed that LMP-1 (but not EBER) induced IL-8 mainly through the activation of NF-kappaB and partly through AP-1 in NPC model cell lines (Ren *et al.*, 2004). Cyclooxygenase-2 (COX-2) is the key enzyme in the biosynthetic pathway of prostaglandins (PGs) and thromboxanes from arachidonic acid. Overexpression of COX-2 can induce angiogenesis through induction of angiogenic factors such as VEGF and bFGF. EBV encoded LMP-1 can induce COX-2 expression which was involved, at least in part, in the enhanced production of VEGF (Murono *et al.*, 2001).

In conclusion, the experimental findings presented here lend credence to our view that NPC has remarkable metastatic ability correlated with MMP9 that might be induced by EBV infection.

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