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Original contribution

Expression of syndecan-1 (CD138) in nasopharyngeal carcinoma is correlated with advanced stage and poor prognosis☆

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Summary Nasopharyngeal carcinoma (NPC) is an important Epstein-Barr virus–associated head and neck malignancy in Taiwan. Syndecan-1 (CD138) is involved in growth, differentiation, invasiveness, and metastatic potential of certain tumors, but its expression in NPC has never been studied. In this study, detection of expression of syndecan-1 protein and Epstein-Barr virus–encoded latent membrane protein-1 (LMP-1) in primary, recurrent, and metastatic NPC specimens in paraffin sections was performed by immunohistochemistry. The quantity of syndecan-1 messenger RNA in tumor cells was investigated by real-time reverse transcriptase polymerase chain reaction using laser capture microdissection. The results of immunohistochemical staining of syndecan-1 and LMP-1 correlated with clinicopathologic features of NPC. Eighteen (20.9%) of 86 primary, 9 (24.3%) of 37 recurrent, and 15 (44.1%) of 34 metastatic NPC samples were positive for syndecan-1, and 37 (43.0%) primary, 18 (48.6%) recurrent, and 12 (35.3%) metastatic samples were positive for LMP-1 expression. Primary NPCs with syndecan-1 protein expression were more frequently associated with advanced clinical stages and worse 5-year survival rates than those without (P = .015 and P = .0021, respectively). Conversely, the LMP-1 expression did not correlate with tumor stage or prognosis but occurred more often in nonkeratinizing carcinoma than keratinizing squamous cell carcinoma (unpublished observation). The inverse expression of syndecan-1 and LMP-1 was noted in primary NPC specimens (total 4/18 versus 35/68, P = .05). The reverse transcriptase polymerase chain reaction revealed low syndecan-1 messenger RNA levels in both primary and metastatic NPC. In conclusion, the protein expression of syndecan-1 in 21% of primary NPC was associated with advanced disease and poor prognosis, and the protein expression correlated with transcription levels.

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1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Taiwan [1]. Most patients have stage III or more advanced disease at the time of diagnosis [1]. Nasopharyngeal carcinoma can be cured by radiotherapy alone or combined chemoradiotherapy, but recurrent or distant metastasis is common and the outcome of patients with recurrent or metastatic NPC remains poor. Still, with primary tumors, the initial 5-year survival rate is approximately 60% to 70% [1,2].

Nasopharyngeal carcinoma is an Epstein-Barr virus (EBV)–associated malignancy that expresses a limited number of latent EBV genes, including Epstein-Barr nuclear antigen-1, latent membrane protein-1 (LMP-1), latent membrane protein-2A (LMP-2A), EBV-encoded RNAs, and *Bam*HI-A rightward transcripts [3]. Latent membrane protein-1 can induce the expression of epithelial growth factor receptor in epithelial cells [4]. Also, LMP-1 has been shown to possess potent tumorigenic effect and promote tumor cell metastasis [5,6].

Epithelial malignancy metastasis and tumor invasion are a multistep process involving several crucial events: the loosening of intercellular junctions, attachment of tumor cells to the extracellular matrix (ECM), degradation of the ECM, migration of tumor cells through the ECM, angiogenesis, detachment of tumor cells, vascular permeation, the homing of tumor cells and trafficking of cancer cells through blood vessels, extravasations, organ-specific homing, and growth [7,8]. Numerous molecules are involved in tumor invasion and metastasis, particularly heparin sulfate proteoglycans [7,8], which can interact with various effector molecules, such as ECM molecules and growth factors [9], and are modulators of cell growth and differentiation. Syndecans belong to a family of cell surface proteoglycans that associate with the actin cytoskeleton to help maintain the morphology of epithelial sheets [10,11].

Syndecan-1, also known as CD138, is the most extensively studied member of the syndecan family. The extracellular domain of syndecan-1 binds both growth factors and ECM components, whereas its cytoplasmic portion interacts with cytoskeletal components [10]. Syndecan-1 expresses mainly in epithelial cells, and its expression is up-regulated during embryonic development [12,13]. Syndecan-1 is thought to be involved in the processes of cell growth, differentiation, and adhesion, and it acts as a coreceptor for fibroblast growth factors, potent angiogenic growth factors involved in differentiation [9,14-16]. The expression of syndecan-1 appears to be generally down-regulated in human carcinomas and in experimental cancer models, whereas transfectional expression of syndecan-1 in cultured cancer cells has been shown to inhibit their growth and other aspects of malignant behavior [17]. Loss of expression of syndecan-1 in tumor cells leads to decreased intercellular cohesion, increased potential for tumor invasiveness, and metastatic spread [18]. However, the expression of syndecan-1 is associated with poor prognosis in some malignancies, such as breast carcinoma [19].

This study examined the expression of syndecan-1 and LMP-1 in primary, recurrent, and metastatic NPC specimens using immunohistochemical staining and analyzed the messenger RNA (mRNA) quantity of the *syndecan-1* gene using real-time reverse transcriptase polymerase chain reaction (RT-PCR) in primary and metastatic NPC tumor tissues using laser capture microdissection on frozen sections of NPC samples. The results correlated with histologic types and clinical data, including age, sex, clinical stage, and outcome, in patients with primary NPC. The interrelationship between expression of syndecan-1 and LMP-1 in NPC was also clarified.

2. Materials and methods

2.1. Pathologic samples

In total, 157 formalin-fixed paraffin-embedded tissue samples from primary (n = 86), recurrent (n = 37), and metastatic (n = 34) NPC tumors were obtained from the Department of Pathology, National Taiwan University Hospital (NTUH), Taipei, Taiwan. Histopathologic classification of primary and recurrent NPC samples was based on the "Pathology and genetics of head and neck tumours" by the World Health Organization in 2005 [20]. The staging system in this study was adapted from the International Union Against Cancer in 1997 [21]. Patients with primary NPC received radiotherapy, and those with recurrent NPC received radiotherapy and/or chemotherapy for treatment. For patients with metastatic NPC, metastatic lesions in the neck, lung, or liver were excised. All patients were followed up at NTUH.

2.2. Immunohistochemical study

Serial paraffin sections were cut to 6 μm in thickness for immunohistochemical studies of the expression of syndecan-1 and LMP-1 in NPC tumor cells. The paraffin sections were baked briefly at 60°C and then deparaffinized and rehydrated using descending alcohol. After antigen retrieval with a 0.01-mol/L citrate buffer at pH 6.0, these sections were incubated with monoclonal antibody against syndecan-1 (CD138) (clone B-B4; Serotec, Oxford, UK) at a 1:100 dilution and LMP-1 (clone CS1-4; Dako, Carpinteria, Calif) at a 1:50 dilution, followed by adequate linked antibody (LsAB; Dako, Carpinteria, Calif). For detecting syndecan-1, the reaction was colorized by diaminobenzidine using a standard indirect avidin-biotin-peroxidase complex, and for LMP-1, the reaction was colorized by new fuchsin using an indirect avidin-biotin-alkaline phosphatase method. Then the sections were counterstained with Mayer's hematoxylin solution. The immunohistochemical staining results were arbitrarily classified into 4 scores depending on the intensity of immunoreactivity: 0, negative staining; 1+, less than 10%

tumor cells with positive immunostaining; 2+, 10% to 50% tumor cells with positive immunostaining; and 3+, greater than 50% tumor cells with positive immunostaining.

For syndecan-1, plasma cells in tissues with chronic inflammation were used as the external positive control, and plasma cells in stroma and nontumor squamous or respiratory cells in tumor tissues were used as the internal positive control. The staining pattern of syndecan-1 was membranous on these cells. The reaction pattern of LMP-1 was cytoplasmic in the NPC tumor cells. The EBV-LMP-1–positive and EBV-LMP-1–negative controls included B95-8 and BJAB lymphoblastoid cell lines, respectively.

2.3. Quantification syndecan-1 mRNA by laser capture microdissection and real-time RT-PCR

Eight freshly frozen NPC samples, including 4 primary and 4 metastatic samples, were available for analyzing the mRNA of *syndecan-1* gene in this study. Laser capture microdissection by AutoPix (Arcturus, Mountain View, Calif) was used to dissect tumor cells from primary and metastatic NPC-frozen sections to study the quantity of *syndecan-1* gene mRNA in the tumor cells. The primer sequences of the *syndecan-1* gene in the current study were published previously [22] and were sense 5′-GAG GGC TGC TGA GGA TGG A-3′ and antisense 5′-ATT CTC CCC CGA GGT TTC AA-3′. The *hypoxanthine phosphoribosyltransferase* (*HPRT*) gene was used as an internal control. The primer sequences of the *HPRT* gene for real-time RT-PCR in the current study were sense 5′-TGA CAC TGG CAA AAC AAT GCA-3′ and antisense 5′-GGT CCT TTT CAC CAG CAA GCT-3′.

Real-time RT-PCR was carried out on an ABI Prism 7700 (Perkin-Elmer/Applied Biosystems, Foster City, Calif), using SYBR green as a detection dye. Conditions for PCR included 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extension). The relative mRNA amount of the target gene/internal control gene (HPRT) was calculated using the CT method as follows: relative expression = 2^{-CT}, where CT = C_T (target gene) − C_T (HPRT).

2.4. Correlation study of expression of syndecan-1 and LMP-1 and clinical data of patients with primary NPC

The results of syndecan-1 and LMP-1 protein expression by immunohistochemistry and *syndecan-1* gene mRNA by

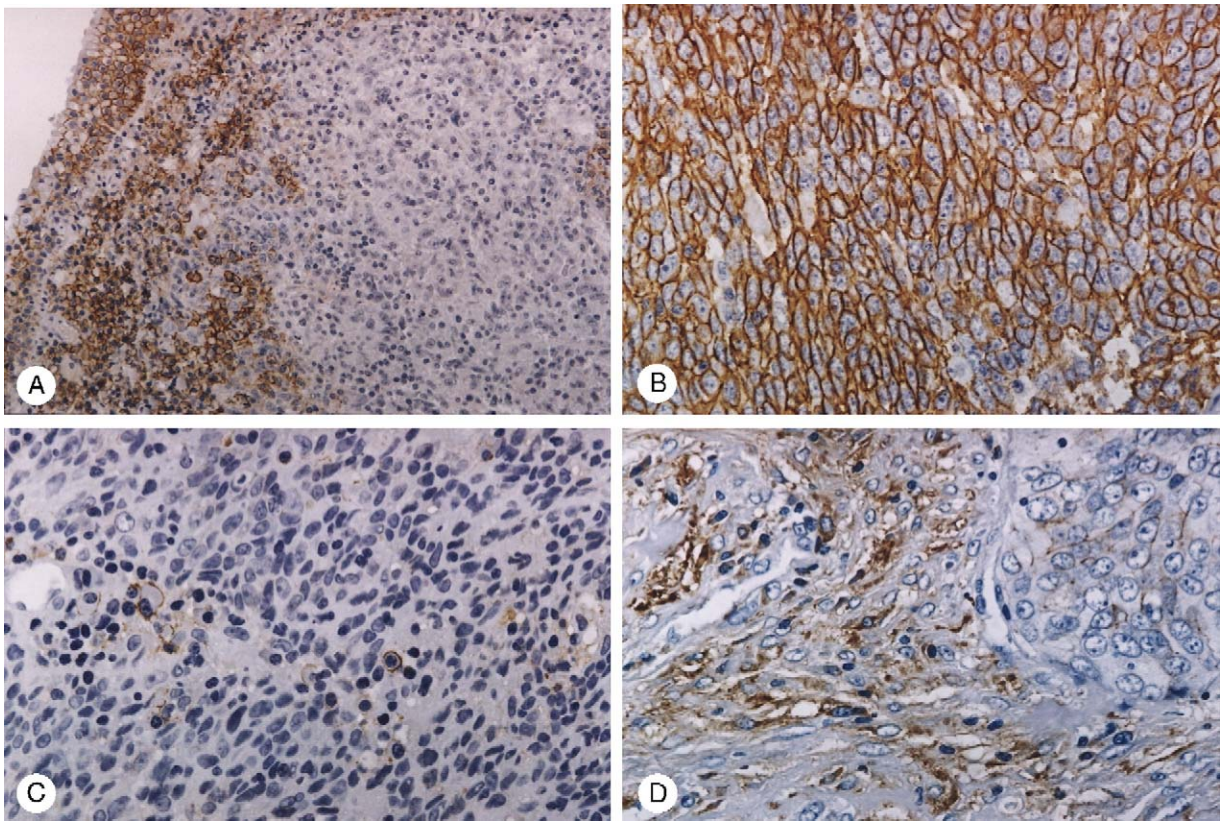

Fig. 1 The syndecan-1 immunostaining in the metaplastic squamous epithelium of nasopharyngeal tissue is typically a strongly positive membrane staining through the full thickness of the layer of squamous cells (upper left) (A). In addition, plasma cells in the stroma also show membranous positivity. In nonkeratinizing NPC cells, syndecan-1 reactivity is quite variable, ranging from (B) strongly diffuse to (C) negative. Only 6 metastatic NPC specimens show positive stromal reactions (D). Images were visualized with immunoperoxidase staining using B-B4 monoclonal antibody with a diaminobenzidine developer and a hematoxylin counterstain.

real-time RT-PCR in patients with primary NPC were correlated with presenting clinical factors, including age, sex, clinical stage, outcomes, and histologic classification of the studied patients. The clinical stage and follow-up data of the patients studied were collected from the medical records of the Department of Registration, NTUH. In our series, 70 patients with primary NPC had enough clinical data available for staging analysis. For statistical analyses, only primary patients with NPC were selected. Univariate statistical analysis was performed using χ^2 tests. The survival curve of patients positive and negative for syndecan-1 was estimated by Kaplan-Meier analysis.

3. Results

3.1. Clinical data

In total, 157 NPC samples were included in this study. Of these, 86 samples were from primary NPC. Patients with primary NPC included 62 men and 24 women whose ages ranged from 18 to 81 years. These primary patients with NPC were histologically classified as follows: 7 with keratinizing squamous cell carcinoma (KSCC) and 79 with nonkeratinizing carcinoma (26 were the differentiated type and 53 were the undifferentiated type).

3.2. Expression of syndecan-1 and LMP-1

In the studied cases, metaplastic squamous cells are strongly positive for syndecan-1 and are used as internal control (Fig. 1A). Regarding the expression of syndecan-1 by immunohistochemical staining in formalin-fixed paraffin-embedded sections of 157 NPC specimens, 18 (20.9%) of 86 primary, 9 (24.3%) of 37 recurrent, and 15 (44.1%) of 34 metastatic specimens stained positively (Fig. 1B and C). The staining intensity of syndecan-1 in tumor cells was usually weaker than in squamous cells of skin or plasma cells in the NPC specimens. In addition, stromal tissue in primary and recurrent NPC specimens did not show any positive staining for syndecan-1, but 6 of the metastatic NPC samples showed positively staining stromal tissue (Fig. 1D), whereas the tumor tissue was negative in 2 of them.

Regarding the expression of LMP-1 in NPC, 37 (43.0%) of 86 primary, 18 (48.6%) of 37 recurrent, and 12 (35.3%) of 34 metastatic specimens stained positively (Fig. 2A and B). The staining pattern in NPC tumor cells was cytoplasmic stippling but was not membranous. The infiltrating lymphocytes in both tumor and overlying epithelium in all samples were negative.

In the LMP-1–positive group, 4 of 37 primary, 3 of 18 recurrent, and 5 of 12 metastatic NPC tissues had coexpression of syndecan-1 in tumor cells, whereas in LMP-1–negative group, 14 of 49 primary, 6 of 19 recurrent, and 10 of 22 metastatic NPC samples expressed syndecan-1. In contrast, in the syndecan-1–positive group, 4 of 18 primary, 3 of 9 recurrent, and 5 of 15 metastatic NPC specimens coexpressed LMP-1 in tumor cells, and in the syndecan-1–negative group, 33 of 68 primary, 15 of 28 recurrent, and 7 of 19 metastatic NPC samples expressed LMP-1.

The real-time RT-PCR study of the *syndecan-1* gene mRNA revealed a low amount of syndecan-1 mRNA in 4 primary NPC and 4 metastatic NPC specimens as compared with the internal housekeeping (*HPRT*) gene. The *HPRT* gene mRNA was detected as early as 28 cycles, but the syndecan-1 mRNA was detected only after 35 to 37 cycles.

3.3. Clinicopathologic correlation of expression of syndecan-1 and LMP-1

The statistical significance of syndecan-1 and LMP-1 expression was analyzed in regard to the clinical data, including age, sex, clinical stage, survival, and histologic types of primary tumors in patients with NPC. Statistical significance was observed in NPC specimens expressing syndecan-1 when correlated with clinical stage and survival of patients (Fig. 3). Syndecan-1 was expressed more often in

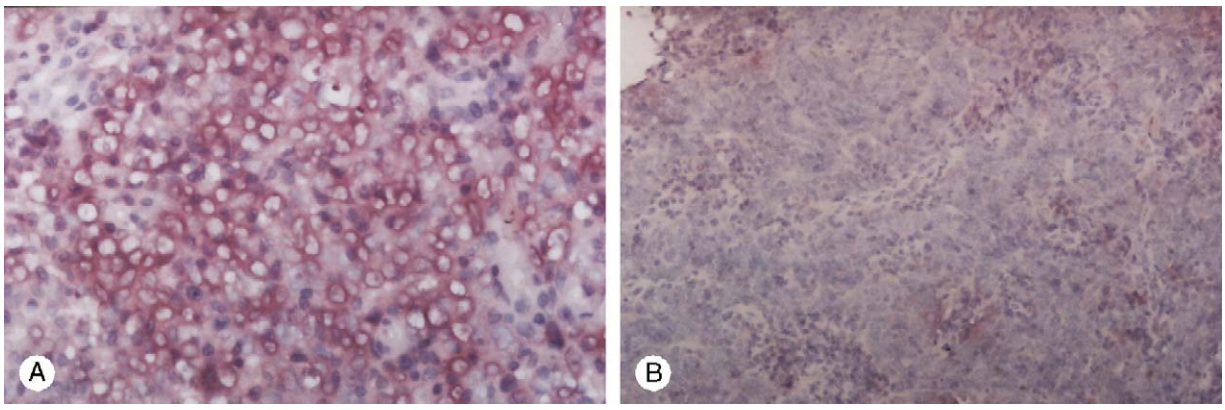

Fig. 2 Epstein-Barr virus–encoded LMP-1 immunostaining in NPC tumor cells of nonkeratinizing undifferentiated type shows positive cytoplasmic staining (A) and negative staining (B). Images were visualized with immunoalkaline phosphatase staining with CS1-4 monoclonal antibody with new fuchsin development and a hematoxylin counterstain.

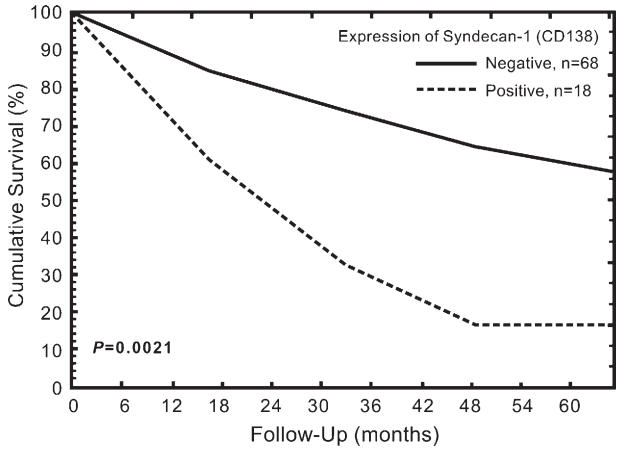

Fig. 3 Kaplan-Meier analysis of the overall survival curves of syndecan-1 (+) and syndecan-1 (−) cases in 86 patients with primary NPCs with statistical significance.

patients at stage III or IV (P = .015) and in patients who survived less than 5 years (P = .0021). The expression of syndecan-1 in NPC tumor specimens did not correlate with age, sex, and histologic types of patients.

The expression of LMP-1 in primary NPC samples did not correlate with age, sex, clinical stage, and patient survival rates, but it did correlate with histologic type of NPC samples with less expression of LMP-1 in KSCC cases than in nonkeratinizing carcinoma.

Statistical analysis of syndecan-1 and LMP-1 expression in tumor tissues of primary versus recurrent or primary versus metastatic NPC specimens revealed considerable differences between primary and metastatic NPC, but no significant difference in syndecan-1 expression in primary versus recurrent NPC or LMP-1 expression in primary versus recurrent or primary versus metastatic NPC specimens.

Regarding the coexpression of syndecan-1 and LMP-1 in NPC specimens, statistical significance was noted in primary NPC samples but not in recurrent or metastatic specimens (P = .05).

4. Discussion

The significance of syndecan-1 expression has been studied in many malignancies, and inverse significance has been found in different tumors. For example, expression of syndecan-1 correlates with the differentiation of tumor cells, low clinical stage, and favorable prognosis in carcinomas of the head and neck regions [23], esophagus [24], larynx [25], liver [26], lung [27], colon [28], and uterine cervix [29], whereas inverse results are noted in malignancies of the breast [19], prostate [30], and thyroid [31].

Because syndecan-1 expression has prognostic significance in squamous cell carcinoma of the head and neck [23], this study examined the expression of syndecan-1, CD138, and EBV-encoded LMP-1 of EBV in primary, recurrent, and metastatic NPC specimens by immunohistochemical stain, analyzing the quantity of mRNA of *syndecan-1* gene via real-time RT-PCR. To the best of our knowledge, this is the first study of syndecan-1 expression and its clinicopathologic significance in NPC.

The current results show that syndecan-1 expression in NPC tumor cells is uncommon, and that the expression level is low in most tumors expressing syndecan-1. These results might correlate with poorly differentiated or undifferentiated NPC tumor cells because, morphologically, most NPC tumors are composed of poorly differentiated or anaplastic tumor cells [32]. However, when the results of syndecan-1 expression and histologic types of primary NPC specimens were correlated, no significant difference was observed. Instead, and surprisingly, the expression of syndecan-1 in NPC samples was statistically related to high clinical stages and poor outcomes for patients with primary NPC. These results were in contrast to those of squamous cell carcinomas of other head and neck regions in which syndecan-1 expression indicates a better-differentiated tumor and a more favorable outcome [23].

Our data suggest that syndecan-1 expressed by NPC tumor cells may play a role in the progression of NPC. The reason for this inverse prognostic implication of syndecan-1 in NPC and other head and neck squamous cell carcinomas is not known but may contribute to the known role of syndecan-1 in tumor progression. Syndecan-1 is known to interact with heparin-binding growth factors and fibroblast growth factors, which are known angiogenic and mitogenic growth factors for many tumors [9,14-16]. It could be hypothesized that high syndecan-1 expression in NPC may confer a particularly important growth advantage by enhancing the response to the other growth factors [19].

To elucidate the possible mechanism of this uncommon syndecan-1 expression in NPC samples, we analyzed the quantity of mRNA of the syndecan-1 gene via real-time RT-PCR. These data support the immunohistochemical results, showing that the quantity of *syndecan-1* gene mRNA was low in most NPC specimens when compared with the amount of mRNA of housekeeping gene. We concluded that this down-regulation of syndecan-1 expression in primary and metastatic NPC was at the transcription level. These results were similar to a previous report by Fujiya et al [28], who found that down-regulation of syndecan-1 in colon carcinomas is due to hypermethylation of the *syndecan-1* gene.

Recent experimental studies surrounding the role of syndecan-1 in malignant transformation have shown that syndecan-1 expression is associated with the maintenance of epithelial morphology, anchorage-dependent growth, and inhibition of invasiveness in vitro [15,16]. The down-expression of syndecan-1 in primary and recurrent NPC might lead to tumor cells having a higher potential for early metastasis to regional lymph nodes or other visceral organs as noted in patients with NPC clinically. However, no

statistical significance was noted in the expression of syndecan-1 in primary and recurrent NPC or between primary and metastatic NPC, although there was a higher percentage of syndecan-1 expression in metastatic NPC samples. The reason for this higher syndecan-1 expression in metastatic NPC samples was not clear.

Induced expression of syndecan-1 in stromal tissue of different types of malignancy has been reported [18,31]. The induced expression of syndecan-1 in stromal tissue might stimulate the growth of epithelial cells and also contribute to tumor cell invasion and development of metastasis [33,34]. In our study, expression of syndecan-1 in stromal tissue was not found in primary or recurrent NPC specimens, but 6 samples of metastatic NPC had syndecan-1 expression in stromal cells, and the tumor cells were negative in 2 cases.

Epstein-Barr virus–encoded LMP-1 has been found to have potent tumorigenic effect in epithelial cells, and it can promote the metastasis of NPC tumor cells [5,6]. The up-regulation of matrix metalloprotease 9 correlates with the metastatic potential in NPC, and the up-regulation of matrix metalloprotease 9 in NPC is associated with the expression of LMP-1 [5]. Latent membrane protein-1 can also induce expression of interleukin 8 through the nuclear factor κB pathway, resulting in angiogenesis in NPC tumors [35]. In the current study, expression of LMP-1 was detected in primary, recurrent, and metastatic NPC, and the overall detection rate of LMP-1 in our NPC specimens was lower than previous reports. This might be due to inadequate processing of the tissue samples before immunohistochemical study.

The expression of LMP-1 in all primary NPC specimens classified as KSCC was negative, whereas 37 of 79 NPC samples classified as nonkeratinizing carcinoma were positive. There was a statistical significance when correlating the histologic types of primary NPC and the status of LMP-1 expression. When analyzing the expression of LMP-1 in primary NPC samples and clinical data, including age, sex, clinical stage, and outcomes of patients with primary NPC, no statistical significance was found. In addition, there was no statistically significant difference between the LMP-1 expression between primary and recurrent NPC or between primary and metastatic NPC.

In regard to the interrelationship of expression of syndecan-1 and LMP-1 in NPC, coexpression of syndecan-1 and LMP-1 was noted in this study, which indicates that the expression of syndecan-1 in NPC might be induced by the expression of LMP-1 in tumor cells and that the expression of both molecules might result in angiogenesis and aggressive behavior of NPC tumors.

In conclusion, in the present work, we studied 2 important molecules in tumorigenesis, angiogenesis, and metastatic ability in NPC tumor cells: syndecan-1 and EBV-encoded LMP-1. We found that syndecan-1 is uncommonly expressed in NPC samples, that its expression correlates with advanced clinical stages and poor outcomes, and that the down-regulation of syndecan-1 in NPC specimens is at the transcription level. Syndecan-1 expression in NPC tumors did not correlate with age and sex of the patients and did not correlate with the histologic types of the NPC samples. A certain proportion of NPC specimens expressed LMP-1, and the KSCC samples were usually LMP-1 negative. The expression of LMP-1 in the NPC samples correlated to the histologic types of the primary NPC specimens but had no statistically significant correlation with age, sex, clinical stage, or survival of patients with primary NPC. The expression of syndecan-1 in primary NPC correlated to the expression of LMP-1.

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