Differential expression of Epstein-Barr virus-encoded RNA and several tumor-related genes in various types of nasopharyngeal epithelial lesions and nasopharyngeal carcinoma using tissue microarray analysis

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Summary  Studies have revealed that Epstein-Barr virus (EBV) infection, genetic aberration, and environmental factors are of importance in the development of nasopharyngeal carcinoma (NPC), although the definite mechanism remains to be fully elucidated. The aim of our study is to investigate using tissue microarray analysis whether differential expression of EBV-encoded small RNA-1 (EBER-1) and several tumor-related genes were associated with NPC carcinogenesis. Immunohistochemistry and in situ hybridization were performed on tissue microarrays containing 148 NPCs and 164 noncancerous nasopharyngeal epithelia (NPE) with different morphologic features. We found that overexpressions of EBER-1 hybridization signals, p53, p21ras, and bcl-2 proteins and loss expressions of p16 and p27 proteins were significantly increased in NPC tissues compared with normal NPE and hyperplastic NPE ($P < .001$). The overexpressions of EBER-1 and p53 ($P < .001$) and the loss expressions of p16 and p27 ($P = .005$) were also significantly higher and more frequently observed in NPC than in dysplastic NPE. The positive expression of EBER-1 hybridization signals in NPC had significant associations with overexpressions of p53 ($P < .001$), p21ras ($P = .041$), and bcl-2 proteins ($P < .001$) and loss expression of p16 protein ($P = .001$). Further analysis confirmed that the abnormal expression of p53, p16, and p27 proteins occurred in the earliest stage of nasopharyngeal epithelial carcinogenesis. In the final logistic regression analysis model, the positive hybridization signals of EBER-1 and the abnormal expression of

Keywords:
Tissue microarray; Nasopharyngeal carcinoma; Epstein-Barr virus; Carcinogenesis

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1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy that occurs at high incidence in southern China and Southeast Asia [1]. NPC derives from the multipotential stem cells of nasopharyngeal mucosa epithelium, such as the reserved cells of columnar epithelium and the basal cells of squamous epithelium [2]. The squamous epithelial metaplasia, epithelial dysplasia, carcinoma in situ, microinvasive carcinoma, and invasive carcinoma are the sequentially morphologic changes found in nasopharyngeal epithelial carcinogenesis [2-7]. The tumorigenesis of NPC is thought to be a multistep process and involves multiple genetic and epigenetic changes [8-12]. NPC has been reported to be strongly associated with the Epstein-Barr virus (EBV) [6,9,10,13,14]. In addition, growing evidences demonstrate that alterations of some oncogenes and tumor suppressor genes are important factors in the multistep process of nasopharyngeal carcinogenesis [9,13-21]. The p53 gene, a well-known tumor suppressor gene, is rarely mutated in NPC. However, high expression of p53 protein revealed by immunohistochemistry (IHC) in primary tumors suggests that abnormal p53 stabilization plays a key role in NPC, particularly in the nasopharyngeal epithelium with EBV infection [6,15,17]. Members of the ras gene family are structurally related and code for a protein (p21) known to play an important role in the regulation of normal signal transduction and cell growth [21,22]. Ras oncogene protein (p21) can induce the transformation of human solid tumors [22]. Overexpression of Ha-ras, rather than mutations, may be an important factor in the development and progression of head and neck tumors [21]. The bcl-2 oncogene inhibits apoptosis and implicates in both differentiation of normal keratinocyte and pathogenesis of epithelial malignancy [18,23]. Tsai et al [24] suggested that the expression of bcl-2 might be one potentially useful biomarker for predicting the outcome of radiotherapy in an NPC patient. The p16 and p27, two important tumor suppressor genes, are frequently inactivated in NPC; hence, they may have significant correlation with the development and progression of NPC [5,8,10,16,17,19,20]. PTEN encodes a dual-activity phosphatase and has been involved in both cell cycle regulation and cell adhesion including migration [25]. Loss of PTEN/p27 expression is associated with adverse pathological parameters, increases in cell proliferation, and higher occurrence of human prostate cancer [26 27]. To our knowledge, the present report is the first to study the association between expression of PTEN protein and NPC clinicopathologic features using tissue microarrays (TMAs).

However, the reports mentioned above, the associations between EBV infection status, the abnormalities of some tumor-related genes, and NPC mainly come from research on a limited number of NPC cases by different research groups [4,7,10,16,18-20,23,24]. Therefore, a study on a larger scale is needed to identify their roles and to evaluate their implications in the multistep process of nasopharyngeal carcinogenesis. Such a comprehensive study is difficult to carry out if it is done by in situ hybridization (ISH) and IHC in conventional full tissue sections from thousands of samples. Recently, the TMAs technology, which speeds the laboratory work and decreases the cost of reagents, is an ideal tool for high-throughput molecular phenotype analysis and rapid evolution of novel histochemical markers in numerous tissues [28,29]. Up to the present, TMAs have been extensively used to study gene targets and potential prognostic markers found by cDNA microarrays and other techniques [28-30], but there is a rare report about its applications in NPC [31].

In the present study, we constructed the NPC-specific TMAs containing NPC and various noncancerous nasopharyngeal epithelia (NPE) with different morphologic features. We took advantage of the high-throughput TMAs technology to investigate the EBV infection and immunophenotypic alterations of p53, p21ras, bcl-2 p16, p27, and PTEN genes in the NPC-specific TMAs. The EBV infection latent status was defined by detecting EBV-encoded small RNA-1 (EBER-1) using ISH, and the expression patterns of p53, p21ras, bcl-2, p16, p27, and PTEN proteins were analyzed by IHC. Our present study should shed light on the roles of the EBV and the tumor-related genes in nasopharyngeal carcinogenesis.

2. Material and methods

2.1. Nasopharyngeal biopsy specimens

Nasopharyngeal biopsy specimens were collected in the ENT department at XiangYa Hospital (Changsha, PR China) during May 2001 and August 2003; there were 148 NPCs, 168 noncancerous NPE including 68 dysplastic NPE, 45 hyperplastic NPE that were nasopharyngeal...
squamous metaplastic epithelia with simple hyperplasia, and 55 histologically normal NPE that were obtained from individuals without evidence of malignancy. Hyperplastic NPE and dysplastic NPE specimens were biopsied from patients with chronically inflamed nasopharyngeal tissues and adjacent noncancerous nasopharyngeal tissues. Of the 148 NPC patients, 93 were male and 57 were female (mean age, 45 years); of the 168 non-NPC patients, 103 were male and 65 were female (mean age, 41 years). All biopsies immediately were fixed in 4% buffered paraformaldehyde, routinely processed, and embedded with paraffin. The immunohistochemical staining for leukocyte common antigen and cytokeratin was performed in 18 cases for differential diagnosis between the NPC and malignant lymphoma. Using WHO histological classification of the NPC and the TNM classification of malignant tumors [32,33], the NPC histological patterns and clinical T stages were classified as follows: 127 cases of differentiated nonkeratinizing carcinomas (NKC), 17 cases of undifferentiated carcinomas (UC), and 4 cases of keratinizing squamous cell carcinomas (KSCC); 45 cases of stage T1, 50 cases of stage T2, 38 cases of stage T3, and 15 cases of stage T4. Of all patients included in the study, 100 patients had cervical lymph node metastasis (n1 = 78, and n2 = 22), and 48 patients had cervical lymph node negative (n0 = 48). No case revealed distant metastasis, hence all of the cases were classified as M0. The hyperplastic NPE and dysplastic NPE were diagnosed and graded by two independent observers under the light microscopy according to the criteria described in the literature [2-5,7,10], including cytological atypia and architectural disarray. The hyperplastic lesions were marked by thickening of the epithelial layer, without loss of normal stratification and morphologic features. The dysplastic lesions were shown by thickening of the epithelial layer, with a loss of normal stratification, exhibiting enlarged polymorphic, hyperchromatic nuclei and distinct nucleoli. The level of atypical cells was used to determine the degree of dysplasia (mild dysplasia—lower third, moderate dysplasia—middle third, and severe dysplasia or carcinoma in situ—upper third of the epithelium).

2.2. Construction of TMAs and validation of the arrayed specimens

Representative areas of NPC and various noncancerous NPE were marked on each hematoxylin-eosin (H&E) slide and tissue paraffin block, and the marked areas of tissue paraffin blocks were sampled for the TMAs. The TMAs were assembled with a tissue-arraying instrument (Beecher Instruments, Silver Springs, Md) as described by Kallioniemi et al [29]. Briefly, the instrument was used to create holes in a recipient paraffin block with defined array coordinates. A solid stylet was used to transfer the tissue cores into the recipient block. Three 0.6-mm-diameter tissue cores were taken from each NPC, and two 0.6-mm-diameter tissue cores were taken from each noncancerous NPE. Three
hundred sixteen nasopharyngeal biopsy specimens were distributed in 2 regular-sized paraffin receptive blocks, each containing 390 spots (Fig. 1A). A serial of 5-μm-thick sections were cut with a Leica microtome (RM 2135, Germany) and transferred to adhesive-coated slides using a paraffin tape-transfer system (Instrumedics Inc, USA). One slide from each recipient block was stained with H&E. The remaining slides were covered with thin paraffin and stored at 4°C before IHC or ISH. An overview of the H&E-stained microarray slide is shown in Fig. 1B. Histological validity of the arrayed specimens was identified under the light microscope (Fig. 1C-E). Four specimens including 3 histologically normal NPE and 1 dysplastic NPE were invalid. Finally, 148 cases of NPC and 164 cases of noncancerous NPE, which contained 67 dysplastic NPE, 45 hyperplastic NPE, and 52 histologically normal NPE, were valid for the present study.

2.3. In situ hybridization for EBER-1

The EBER-1 probe, 5′-AGA CAC GTT CCA CCA CAC CCT GGA TTG GTA-3′, which recognizes a region of the EBV genome transcribed in latently infected cells described in literature [34], was labeled with 11-DIG-dUTP at its 3′ tailing (DIG oligonucleotide 3′ -tail labeling Kit, 2nd Generation, Roche). The ISH detection method has been previously described with minor modifications [35]. Briefly, each TMA section was deparaffinized, rehydrated, digested with 2 μg/mL proteinase K at 37°C for 15 minutes, and dehydrated. The slides were incubated with prehybridization solution at 37 °C for 2 hours and hybridized with EBER-1 probe 37°C overnight. Hybridization was detected by incubation with antidigoxigenin horseradish peroxidase, fab fragments (anti-DIG-POD, Roche Molecular Biochemicals). The color reaction was performed with 3,3′-diaminobenzidine tetrahydrochloride (DAB), and the slides were counterstained with hematoxylin. A poly d (T) was used as a control for total RNA preservation. A known EBV-positive NPC tissue section was used as positive control, and hybridizing without EBER-1 probe was used as negative control. A positive hybridization signal was identified by dark brown staining. The positive hybridization signals of EBER-1 were scored microscopically at 400× magnifications [9]. The scoring was graded as 0 (negative), 1 (<10% nuclei being EBER-1–positive), 2 (10%-50% positive), or 3 (>50% positive) in accordance with the staining proportion and intensity [7]. The final scores were regarded as negative (0 score) and positive (1-3 score).

2.4. Immunohistochemistry

Immunohistochemical staining of all antibodies under the study was performed on the TMAs slides using the streptavidin-peroxidase (S-P) method as previously described with minor modifications [36,37]. The staining conditions for each antibody were adjusted according to previous data from the literature and our laboratory experience. Briefly, each TMA section was deparaffinized
Differential expression of EBER and several tumor-related genes
and rehydrated, and high-temperature antigen retrieval was achieved for all antibodies by heating the samples in 0.01 M citrate buffer (pH 6.0) in a domestic microwave oven at full power (750 W) for 15 minutes, then the samples were immersed into methanol containing 0.3% H2O2 for 30 minutes at 37°C to inactivate endogenous peroxidase. To eliminate nonspecific staining, we incubated the slides with appropriate preimmune serum for 30 minutes at room temperature, followed by incubation with primary antibodies at 4°C overnight. These antibodies included monoclonal mouse antihuman p53, 1:100 (clone DO-7, Dako); p21ras, 1:100 (clone NCC-RAS-001, Dako); bcl-2, 1:100 (clone 100/D5, Neomarkers); p16, 1:100 (clone F-12, Santa Cruz); p21, 1:100 (clone F-8, Santa Cruz); and PTEN, 1:100 (clone 28H1, Lab Vision). Secondary antibody (biotinylated goat antimouse IgG) and streptavidin-peroxidase conjugate (S-P kit, Dako) were added according to the manufacturer’s instructions. Color reaction was developed using DAB chromogen solution (DAB Liquid, Dako), and all slides were counterstained with hematoxylin. Positive and negative control slides (according to manufacturer’s data sheet of each antibody) were included in every experiment in addition to the internal positive controls.

Immunohistochemical staining of TMA sections were scored microscopically at 400× magnification in all available tumor or epithelial cells meeting the typical morphologic criteria by 2 pathologists using the qualitative scale described in literatures with minor modifications [36,37]. Each specimen was represented on the arrays by at least one core. Disc scores from the same specimen were averaged to produce a single score. The number of cells staining was scored as 0 (no staining), 1 (<1/3 positive cells), 2 (>1/3 and <2/3 positive cells), and 3 (>2/3 positive cells), and immunostaining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The sum of the intensity and staining extent score was used as the final immunoreactive score (0-6). The final scores were regarded as negative (0 score) and positive (1-6 scores). To adapt immunohistochemical scores for the unsupervised hierarchal cluster analysis [30], our usual scoring system also designates 0 = negative (0 score), 1 = weak positive (1-2 scores), 2 = moderate positive (3-4 scores), and 3 = strong positive (5-6 scores). Nuclear staining of more than 2% cells for p53 was considered to be positive according to previous literature [36].

### 2.5. Hierarchal cluster analysis

Hierarchal cluster analysis of our TMA data was performed using software tools, which were designed by Liu et al [30]. All scores were input into one standardized electronic spreadsheets (Excel for Windows) as described by Liu et al [30]. The spreadsheets were processed using the TMA-Decovulter 1.07 (http://genome-www.stanford.edu/TMA/explore.shtml), the Cluster 3.0 and TreeView 1.60 programs (http://rana.lbl.gov/EisenSoftware.htm) were adapted for TMA analysis [30].

### 2.6. Statistical analysis

For the analysis of the association of the different variables with nasopharyngeal carcinogenesis, we used Fisher exact test for nonparametric variables where applicable. Spearman correlation test was used to evaluate the pairwise association between abnormal expressions of

![Table 1](http://example.com/table1.png)

**Table 1** Association between abnormal expression of EBER-1 hybridization signals and the analyzed genes proteins and NPC clinical pathological features (N = 148)

<table>
<thead>
<tr>
<th>Histological types</th>
<th>EBER-1 (%)</th>
<th>p53 (%)</th>
<th>p21ras (%)</th>
<th>bcl-2 (%)</th>
<th>p16 (%)</th>
<th>p27 (%)</th>
<th>PTEN (%)</th>
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<tbody>
<tr>
<td>NKC (n = 127)</td>
<td>112 (88.2)</td>
<td>97 (76.4)</td>
<td>117 (92.1)</td>
<td>105 (82.7)</td>
<td>29 (22.8)</td>
<td>37 (29.1)</td>
<td>102 (80.3)</td>
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<tr>
<td>UC (n = 17)</td>
<td>16 (94.1)</td>
<td>13 (76.5)</td>
<td>16 (94.1)</td>
<td>15 (88.2)</td>
<td>2 (11.8)</td>
<td>4 (23.5)</td>
<td>13 (76.5)</td>
</tr>
<tr>
<td>KSCC (n = 4)</td>
<td>2 (50.0)</td>
<td>2 (50.0)</td>
<td>3 (75.0)</td>
<td>2 (50.0)</td>
<td>2 (50.0)</td>
<td>2 (50.0)</td>
<td>3 (75.0)</td>
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<table>
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<tr>
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<td>.082</td>
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<td>.299</td>
<td>.153</td>
<td>.238</td>
<td>.582</td>
<td>1.000</td>
</tr>
<tr>
<td>.080</td>
<td>.544</td>
<td>.352</td>
<td>.148</td>
<td>.148</td>
<td>.544</td>
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<table>
<thead>
<tr>
<th>T stages</th>
<th>EBER-1 (%)</th>
<th>p53 (%)</th>
<th>p21ras (%)</th>
<th>bcl-2 (%)</th>
<th>p16 (%)</th>
<th>p27 (%)</th>
<th>PTEN (%)</th>
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</thead>
<tbody>
<tr>
<td>T1-2 (n = 95)</td>
<td>84 (88.4)</td>
<td>65 (68.4)</td>
<td>88 (92.6)</td>
<td>73 (76.8)</td>
<td>27 (28.4)</td>
<td>31 (32.6)</td>
<td>79 (83.2)</td>
</tr>
<tr>
<td>T3-4 (n = 53)</td>
<td>46 (86.8)</td>
<td>47 (88.7)</td>
<td>48 (90.6)</td>
<td>49 (92.5)</td>
<td>6 (11.3)</td>
<td>12 (22.6)</td>
<td>39 (73.6)</td>
</tr>
<tr>
<td>P</td>
<td>.797</td>
<td>.005</td>
<td>.756</td>
<td>.023</td>
<td>.023</td>
<td>.238</td>
<td>.202</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymph node status</th>
<th>EBER-1 (%)</th>
<th>p53 (%)</th>
<th>p21ras (%)</th>
<th>bcl-2 (%)</th>
<th>p16 (%)</th>
<th>p27 (%)</th>
<th>PTEN (%)</th>
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<tbody>
<tr>
<td>N1-2 (n = 100)</td>
<td>89 (89.0)</td>
<td>74 (74.0)</td>
<td>91 (91.0)</td>
<td>84 (84.0)</td>
<td>18 (18.0)</td>
<td>27 (27.0)</td>
<td>79 (79.0)</td>
</tr>
<tr>
<td>N0 (n = 48)</td>
<td>41 (85.4)</td>
<td>38 (79.2)</td>
<td>45 (93.8)</td>
<td>38 (79.2)</td>
<td>15 (31.3)</td>
<td>16 (33.3)</td>
<td>39 (81.3)</td>
</tr>
<tr>
<td>P</td>
<td>.594</td>
<td>.545</td>
<td>.752</td>
<td>.494</td>
<td>.091</td>
<td>.444</td>
<td>.830</td>
</tr>
</tbody>
</table>

*T1-2 indicates clinical T1 and T2 stage; T3-4, clinical T3 and T4 stage.*

*P* value for Fisher exact test.

a NKC vs KSCC.
b UC vs KSCC.
EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins in NPC. Furthermore, we used standard logistic regression model with 2 categories (NPE and NPC) and with covariates for EBER-1 and 6 analyzed genes to investigate whether NPC carcinogenesis depends on the EBV infection and immunophenotypic alterations of these tumor-related genes. *P* values less than .05 were considered significant. Calculations were performed using SPSS 12.0 statistical software for Windows (SPSS Inc, Chicago, Ill).

3. Results

3.1. Expression profiles of EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins on TMAs containing NPC and various types of noncancerous NPE

EBER-1 ISH signals were observed in the nuclei of NPC cells, nasopharyngeal epithelial cells, and occasionally in limited number of infiltrating lymphocytes (Fig. 2A-B). Representative images of the positive expressions of p53, p21ras, bcl-2, p16, p27, and PTEN proteins were shown in Fig. 3A-H.

Associations between the abnormal expression of EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins and NPC clinicopathological features are shown in Table 1. In comparison, between the differentiated NKC and UC and between differentiated NKC and KSCC, none of the molecular markers were statistically associated with NPC histological types. We only observed the increased trend of positive percentage of EBER-1 in differentiated NKC (*P* = .082) as well as in UC (*P* = .080). However, significant differences were found in the comparison between NPC clinical T1-2 stages and T3-4 stages for overexpressions of p53 (*P* = .005) and bcl-2 proteins (*P* = .023) and loss expression of p16 protein (*P* = .023). In the present study, abnormal expression of EBER-1 as well as p21ras, p27, and PTEN proteins showed no significant differences in NPC clinical T stages and NPC with or without cervical lymph node metastases (*P* > .05 for all).

The pairwise association between abnormal expressions of EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins in NPC is revealed in Table 2. Positive expression of EBER-1 hybridization signals had significant associations with overexpressions of p53 (Spearman, *r* = .183, *P* = .026), p21ras (*r* = .175, *P* = .033), and bcl-2 proteins (*r* = .220, *P* = .007) and loss expression of p16 protein (*r* = .230, *P* = .0005). Similarly, overexpression of p53 showed statistically associations with overexpressions of p21ras (*r* = .781, *P* < .001) and bcl-2 proteins (*r* = .174, *P* = .034) as well as loss expression of p16 protein (*r* = .191, *P* = .020). We also found that overexpression of the bcl-2 protein was evidently associated with positive expression of p21ras (*r* = .155, *P* = .090) and loss expressions of p16 (*r* = .169, *P* = .04). However, overexpressions of EBER-1, p53, p21ras, and bcl-2 and loss expressions of p16 and p27 failed to find significant association with PTEN expression in NPC (*P* > .05 for all).

Abnormal expressions of EBER-1, p53, p21ras, bcl-2, p16, p27, and PTEN proteins in NPC and noncancerous NPE were analyzed; results are summarized in Table 2. In the comparison between NPC and noncancerous NPE,
significant differences were observed in EBER-1 hybridization signals and expressions of p53, p21ras, bcl-2, p16, p27, and PTEN proteins (P < .01 for all), but no statistical difference was found in PTEN (P = .064). In the comparison between various types of noncancerous NPE and NPC for differential expressions of EBER-1, p53, p21ras, bcl-2, p16, p27, and PTEN, results are demonstrated in Fig. 4, significant differences were observed in normal NPE and hyperplastic NPE compared with NPC for EBER-1 hybridization signals and protein expressions of p53, p21ras, bcl-2, p16, and p27 (P < .05 for all). NPC revealed more frequent overexpressions of EBER-1 hybridization signals, p53, p21ras, and bcl-2 proteins (P < .001 for all) and loss expressions of p16 (P < .001) and p27 proteins (P < .001) compared with histologically normal NPE. Similarly, the overexpressions of EBER-1, p53, p21ras, and bcl-2 as well as the loss expressions of p16 and p27 were significantly higher and frequently observed in NPC than in

Fig. 4
Comparison of abnormal expression of EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins between NPC and various types of noncancerous nasopharyngeal epithelium. P value denotes significant differences between the groups statistically evaluated by χ² test.

Fig. 5
Comparison of abnormal expression of EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins among various types of noncancerous nasopharyngeal epithelium. P value denotes significant differences between the groups statistically evaluated by χ² test.
hyperfraction NPE ($P \leq .001$). The overexpressions of EBER-1 and p53 ($P < .001$) and loss expressions of p16 ($P < .001$) and p27 ($P = .005$) were also significantly higher and frequently observed in NPC than in dysplastic NPE. In the present study, abnormal expression of PTNE failed to indicate the significant difference between other various types of nasopharyngeal epithelia and NPC except between histologically normal NPE and NPC ($P = .016$). These results indicated that the gradual accumulation of inactivations of some tumor suppressor genes, the activations of oncogenes, as well as the high infection rate of EBV were associated with the nasopharyngeal carcinogenesis progression pathway through various types of nasopharyngeal epithelial lesions to NPC.

In the various noncancerous NPE lesions, abnormal expressions of EBER-1 as well as p53, p21 ras, bcl-2, p16, p27, and PTEN proteins were compared; results are shown in Fig. 5. In the comparison between hyperplastic NPE and normal NPE, the overexpression of p53 protein but loss expressions of p16 ($P < .001$) and p27 proteins ($P = .019$) were significantly higher and frequently observed in hyperplastic NPE than in normal NPE ($P = .049$). There were evidently higher positive expression of EBER-1 hybridization signals ($P < .001$), p53 ($P = .004$), p21 ras ($P = .004$), and bcl-2 proteins ($P < .001$) and loss expressions of p16 ($P < .001$) and p27 proteins ($P < .001$) in dysplastic NPE than in normal NPE. In addition, positive expressions of EBER-1 hybridization signals ($P < .001$) and bcl-2 protein ($P = .011$) were significantly higher and frequently found in dysplastic NPE than in hyperplastic NPE. None of the noncancerous NPE statistically associated with each other for abnormal expression of PTEN ($P > .05$ for all).

To investigate whether differential expressions of EBER-1 and 6 analyzed genes might serve as new prognostic factors for nasopharyngeal carcinogenesis, we next performed a comprehensive statistical data evaluation and comparison. In the preliminary univariate analysis giving rise to the final logistic regression analysis to evaluate and compare independent contribution of EBER-1 and 6 analyzed genes, the final model is shown in Table 4. The positive hybridization signals of EBER-1 were the most significant, independent predictor of nasopharyngeal carcinogenesis from the noncancerous nasopharyngeal epithelium to NPC, the hazard ratio (HR), given by the expression $\exp(B)$ is 13.412 (95% confidence interval [CI] 6.179-29.111, $P < .001$), along with the overexpression of p53 (HR=3.849, 95% CI 1.794-8.255, $P = .001$), and the loss expressions of p16 and p27 proteins (HR=0.115, 95% CI 0.055-0.240, $P < .001$; HR=0.235, 95% CI 0.118-0.468, $P < .001$, respectively). A univariate analysis identified that the overexpressions of p21 and bcl-2 proteins were the risk factors for nasopharyngeal carcinogenesis (Table 2; Figs. 4 and 5), but the expressions of p21ras and bcl-2 proteins were not significantly associated with nasopharyngeal carcinogenesis in the logistic regression model (Table 4). Expression of PTEN was not a significant predictive factor of nasopharyngeal carcinogenesis in either univariate (Table 3) or multivariate analysis (Table 4).

### 3.2. Cluster analysis of EBER-1 hybridization signals, p53, p21 ras, bcl-2, p16, p27, and PTEN protein expression profiles

The unsupervised hierarchical clustering [30] was used to analyze the data from EBER-1 hybridization signals, p53, p21 ras, bcl-2, p16, p27, and PTEN protein expression

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Comparison between abnormal expression of EBER-1 hybridization signals and the analyzed genes proteins in NPC and noncancerous NPE (N = 312)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>NPC (%)</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>EBER-1</td>
<td>130/148 (87.8)</td>
</tr>
<tr>
<td>p53</td>
<td>112/148 (75.7)</td>
</tr>
<tr>
<td>p21</td>
<td>123/148 (84.9)</td>
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<td>bcl-2</td>
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<td>p16</td>
<td>33/148 (22.3)</td>
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<tr>
<td>p27</td>
<td>43/148 (29.1)</td>
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<tr>
<td>PTEN</td>
<td>118/148 (79.7)</td>
</tr>
</tbody>
</table>

Note: $P$ value for Fisher exact test.

* Noncancerous NPE includes normal NPE, hyperplastic NPE, and dysplastic NPE.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Logistic regression analysis of abnormal expression of EBER-1 hybridization signals and the analyzed genes proteins related to nasopharyngeal carcinogenesis</th>
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<tbody>
<tr>
<td>Variables in the equation</td>
<td><strong>Factor</strong></td>
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<tr>
<td>EBER-1</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
</tr>
<tr>
<td>p21ras</td>
<td></td>
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<tr>
<td>bcl-2</td>
<td></td>
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<tr>
<td>p16</td>
<td></td>
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<td>p27</td>
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<td>PTEN</td>
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</table>
profiles on the NPC-specific TMAs, and the results are presented graphically in Fig. 6. In general, the graphic results showed the nasopharyngeal specimens were clustered into 2 main clusters, and each cluster contained 2 branches with similarity diagnosis. The first cluster is mainly composed of NPC and dysplastic NPE with loss expressions of p16 and p27 proteins, although with overexpressions of p53, p21ras, bcl-2 proteins, and EBER-1 hybridization signals. The second cluster mainly contained hyperplastic NPE and histologically normal NPE with high positive expressions of p16, p27, and PTEN proteins, but with low expressions of p53, p21ras, bcl-2 proteins, and EBER-1. All the analyzed genes were also clustered into 2 main clusters based on abnormal expressions of EBER-1 hybridization signals, p53, p21ras, bcl-2, p16, p27, and PTEN proteins in NPC and various types of noncancerous NPE. The first cluster was composed of p16 and p27 genes, and the second cluster was composed of 3 subgroups including EBV infection (EBER-1) and p53 gene, p21ras and bcl-2 genes, and PTEN gene.

4. Discussion

No human tumor entity is as consistently associated with EBV as NPC [38]. Therefore, it is important to define the
exact timing of EBV infection in nasopharyngeal epithelial cells and to identify the factors that precede and probably pave the way for EBV infection [38]. EBV has been detected by various methods, including immunoblotting, nucleic acid hybridization, ISH, and polymerase chain reaction. Among them, polymerase chain reaction and ISH have been reported as sensitive methods for detecting EBV in NPC, but ISH has the advantage of being able to precisely localize EBV in tumor cells. ISH for the EBERs is regarded as the “gold standard” for the detection of latent EBV infection in clinical tissues being the abundance and stability of EBERs in paraffin-embedded tissue sections. In the present study, EBER-1–positive hybridization signals were significantly higher in NPC than that in the noncancerous NPE, which were consistent with previous reports [4-6,9,10,17,38]. The precise role of EBV in NPC development remains to be clearly defined, but higher EBV latent infection in dysplastic NPE (67.1%) and NPC (87.8%) is revealed in our present study and previous reports [4,5,10]. These findings speculate that the first step that EBV plays in the process of nasopharyngeal carcinogenesis may occur in the dysplastic lesions. Our data support the notion that EBV is not simply a passenger in the process of NPC carcinogenesis. EBV infection may be a critical step for the progression of precancerous lesions to malignant tumors, but it does not represent the first step in NPC tumorigenesis [4-7,9,14].

Our current data revealed that EBER-1 presence had a significantly positive association with overexpressions of p53, p21ras, and bcl-2 proteins and a negative association with p16 protein expression in NPC. These findings confirmed further that NPC carcinogenesis is not simply a consequence of EBV infection, and the development of NPC may involve multiple genetic changes [5,6,9-11,13,17,23]. The p53 gene abnormalities may be extremely important in NPC carcinogenesis [6,15,17,18]. The highest frequencies of loss of heterozygosity on chromosome 3p, 4q, 9p, 11q were found in NPC, dysplasias of the nasopharynx, and NPC reported in literatures [5,8,10]. The overexpression of p53 protein in normal epithelium might be a result of the mutation of p53 gene or accumulation of wide-type p53 protein with altered biological functions [5,6,8,10,15,18]. These findings suggested that such genetic lesions are early events that occur in apparently normal nasopharyngeal epithelium [5,8,10]. In the present study, our data revealed that the overexpression of the p53 protein had the key role in the transition from normal NPE to dysplastic NPE and NPC, and the abnormal expression of p53 protein seemed to occur at an early stage in the process of nasopharyngeal carcinogenesis. In addition, our results indicated that the overexpression of p53 protein had a statistically positive association with expressions of p21ras and bcl-2 proteins in NPC. It is suggested that the abnormal expression of p53 protein might act as an activator for gene transcription of various key oncogenes, such as p21ras and bcl-2, that play an important role in the development of NPC [6,15,17,18,23,24]. In the present study, there were higher positive expressions of p21ras and bcl-2 proteins in dysplastic NPE and NPC, which implicate that bcl-2 and p21ras play crucial role in their pathogenesis [6,15,18]. Moreover, our current data showed that overexpressions of bcl-2 and p53 proteins had a significantly positive association with advanced stages of NPC, which were similar with previous studies [15,17,18,39,40].

Occurrence of NPC usually correlates with loss expression of the tumor suppressor genes, such as p16 and p27, through chromosome deletion and promoter methylation. Inactivation of these tumor suppressor genes leads to deregulated cell proliferation and is a key factor in the development and progression of NPC [9,11,16,19,20].
the present study, loss expressions of p16 and p27 proteins were more often detected in NPC than those in various noncancerous NPE, which is in agreement with previous reports [9,16,20]. Further analysis showed loss expressions of p16 and p27 proteins in hyperplastic NPE were significantly higher than in the normal NPE (Fig. 5). These findings suggested that the inactivation of p16 and p27 genes might be the earliest molecular event of nasopharyngeal epithelial carcinogenesis and might significantly correlate with the development of NPC. Our present data indicated that the loss expression of p16 protein in NPC had statistical associations with overexpression of EBER-1 hybridization signals and p53 protein as well as NPC clinical T stages. These findings imply that the inactivation of the p16 gene gives rise to multiple abnormal development of the nasopharyngeal epithelium including squamous metaplasia, simple hyperplasia, dysplastic hyperplasia, and NPC. Thus, it appears that preneoplastic nasopharyngeal epithelium with the inactivation of the p16 gene becomes susceptible to EBV infection, and EBV may be an essential event in the development of NPC. In the present study, we noticed that the loss of PTEN expression was only statistically significantly higher in NPC than in normal NPE, but there was no clear difference between NPC and dysplastic NPE or hyperplastic NPE. The finding was consistent with the previous reports about PTEN protein expression in sporadic colorectal tumors [41]. Our experimental findings speculate that the loss of PTEN protein may not be prevalent in the progression of nasopharyngeal carcinogenesis. The reason for such observation is unclear and remains to be investigated in further studies.

Our current data of hierarchical clustering showed that nasopharyngeal specimens with similar diagnoses and genes with similar function were clustered into one (Fig. 6). For example, the overexpression of p53 protein and higher EBV infection rate in NPC were well known, as well as the loss expression of p16 and p27 proteins. These findings demonstrated that nasopharyngeal carcinogenesis was associated with abnormal changes of multiple genes [5,6,9-11,13,17,23]. We proposed one simplified model to illustrate that EBV infection and abnormal expressions of p53, p21ras, bcl-2, p16, p27, and PTEN proteins might be involved in the multistep process of nasopharyngeal carcinogenesis based on the present findings using TMAs analysis (Fig. 7). In the earliest stage of carcinogenesis, from normal NPE to hyperplastic NPE, overexpression of the p53 protein and loss expression of p16 and p27 proteins were the major changes. From the stage of histologically normal NPE to dysplastic NPE, molecular abnormalities mainly included high frequency of EBV infection (high expression of EBER-1), overexpression of p53, p21ras, and bcl-2 proteins, and significantly low expressions of p16 and p27 proteins. Molecular alterations from dysplastic NPE to NPC were high EBV infection, overexpression of p53 protein, and loss expressions of p16 and p27 proteins. Our data suggested that abnormal expressions of p53, p16, and p27 proteins might occur in the earliest stage of the nasopharyngeal carcinogenesis process. These findings further proved the scientific hypothesis that early genetic alterations may take place before EBV latent infection [10].

Markers hiring for identifying cancerous lesion is of great practical use in the diagnosis of NPC [6,7,42]. Test for NPC-related antibodies is also valuable for the screening of NPC in patients presenting without relevant symptoms and predicting the prognosis of NPC patients [6,7,9,17,18,20]. The logistic regression model is much more stringent because it has to accommodate all data, thus diluting the statistical power of some correlations, which nevertheless do exist if analyzed individually [43]. In the study, the multivariate analysis showed that the positive hybridization signals of EBER-1, overexpression of p53 protein, and loss expressions of p16 and p27 proteins were independent contributions for nasopharyngeal carcinogenesis, and the EBER-1 was the most significant, independent predictor of nasopharyngeal carcinogenesis from the noncancerous nasopharyngeal epithelium to NPC (Table 4). Results suggested that EBV infection was significantly associated with nasopharyngeal carcinogenesis independently, which EBER-1 might serve as the molecular marker to distinguish NPC from noncancerous NPE.

In summary, the overexpression of p53 protein and loss expressions of p16 and p27 proteins may occur in the earliest stage of the nasopharyngeal carcinogenesis process. EBV infection, together with overexpressions of p53 and loss expressions of p16 and p27 proteins may be involved in the multistep process of human nasopharyngeal epithelial carcinogenesis. The positive hybridization signals of EBER-1 were the most significant, independent predictor of nasopharyngeal carcinogenesis from the noncancerous nasopharyngeal epithelium to NPC. TMAs technology combined with novel analysis methods can enhance the evaluation of the molecular phenotypes changes in the multistep process of nasopharyngeal carcinogenesis.

References

Differential expression of EBER and several tumor-related genes


