Introduction

The Epstein-Barr virus (EBV) is a ubiquitous infectious agent that infects more than 90% of the world’s population [1]. EBV is usually acquired in early childhood in developing countries. In developed countries, primary infection in adolescence is associated with the clinical syndrome infectious mononucleosis. Following primary infection, EBV persists lifelong in the host in a latent state in memory B lymphocytes. The EBV is considered to play a critical role in the progression of nasopharyngeal carcinoma (NPC) from precancerous lesion to malignancy; however, only a small fraction of EBV-infected individuals go on to develop NPC. The incidence of NPC has a rather distinct and geographically well-defined distribution worldwide [2]. It is higher in Southern China, South-east Asia, and to a lesser extent in the Maghreb Arabic regions of Northern Africa, the Arctic and Alaskan Eskimos, but rare in other areas of the world [3,4]. Nevertheless, the strong association between NPC and EBV is observed in all regions evaluated [2,4,5]. While host genetics, diet, and environment may influence the prevalence of EBV-associated NPC in different geographical areas, it...
is also possible that certain EBV strain variations contribute to geographic distribution.

According to sequence divergence within the EBV nuclear antigens (EBNA-2, -3A, -3B, -3C) and the different capacity to transform B lymphocytes into a state of continuous immortalization, EBV can be classified as genotypes A or B, also known as type 1 or 2 \[6\]. Compared with type A, type B has a lower transforming efficiency, a poorer initial outgrowth, and higher cell density dependence for cell viability in vitro \[7\]. It has been reported that the incidence of EBV infection varies geographically \[8\]. Type A is predominant in Southern China, Japan, Tunisian, Slovenia, and North America, whereas type B has been found mainly in Alaska \[3, 9–12\]. A further attempt to identify polymorphisms of virus isolates in the \textit{Bam}HI I and \textit{Bam}HI F region of the EBV genome resulted in the so-called type C/D and type F/\textit{f}, which represent single nucleotide polymorphisms at \textit{Bam}HI sites on the viral genome. Types C and F do not have a \textit{Bam}HI restriction site in the \textit{Bam}HI W1/I1 region and \textit{Bam}HI F region, respectively, while types D and \textit{f} possess this site in the corresponding region. Type C prevails in NPC patients from Southern China, while type D is prevalent in the United States \[9, 13, 14\]. The \textit{f} variant appears to be more frequent in NPC patients from Southern China than in healthy Chinese individuals, suggesting that this variant may be tumor associated \[15, 16\]. The genotypic characterization of EBV in NPC patients from endemic areas of the world, especially Southern China, has already been defined. It has been demonstrated that the predominant combination of EBV genotypes in NPC in Southern China is types A, C or \textit{f} \[9, 15\]. However, little information has been collected to date from Northern China where NPC is nonendemic. The aim of this study is to characterize the specific EBV polymorphisms in virus isolates obtained from NPC patients and healthy donors in Northern China and to explore the relationship between the EBV genotypes and NPC.

### Materials and Methods

#### Clinical Samples

This study was approved by the Medical Ethics Committee at the Medical College of Qingdao University, China. One hundred and seventy-nine formalin-fixed, paraffin-embedded tissue samples of histologically confirmed NPC were obtained from the Department of Pathology of the Affiliated Hospital of the Affiliated Hospital of Qingdao University Medical College and the Affiliated Qilu Hospital of Shandong University Medical College, China. In situ hybridization for EBV-encoded small RNA (EBER) 1 was performed according to methodology described previously \[17\] to confirm the presence of EBV in 140 of 179 (78.2%) NPC tissues. In addition, throat washings (TWs) were collected from healthy adults in the same geographic region by gargling with 15 ml of phosphate-buffered saline. The \textit{Bam}HI W fragment-positive TWs were used as controls. The primers for \textit{Bam}HI W fragment and EBER1 probes are listed.

### Table 1. List of primers and probes used in the present study

<table>
<thead>
<tr>
<th>Name of primers or probes</th>
<th>Sequence (5'–3')</th>
<th>Size of PCR products or after RE digestion</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Bam}HI W primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Bam}HI-1</td>
<td>CCGACAGACGGCCAATTGT</td>
<td>129 bp</td>
<td>41</td>
</tr>
<tr>
<td>\textit{Bam}HI-2</td>
<td>TGAAGACCCCCCTTTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBER1</td>
<td>AGACACCGGTCCTCAACC</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>Antisense probe</td>
<td>CCGGTGGGAGAGTGGGTC</td>
<td>type A = 153 bp</td>
<td></td>
</tr>
<tr>
<td>Sense probe</td>
<td>CCGGTGGGAGAGTGGGTC</td>
<td>type B = 246 bp</td>
<td></td>
</tr>
<tr>
<td>EBNA3C primers</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Sense</td>
<td>AGAAGGGGAGGCTGTGTTG</td>
<td>type C = 206 bp</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GGCTCGTTTTTGAGCTG</td>
<td>type D = 130 + 76 bp</td>
<td>14</td>
</tr>
<tr>
<td>\textit{Bam}HI W1/I1 primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>ACCTGCTACTCTCGGGAAC</td>
<td>type F = 198 bp</td>
<td>14</td>
</tr>
<tr>
<td>Antisense</td>
<td>TCTGTCACACCTCACTGT</td>
<td>prototype \textit{F} = 127 + 71 bp</td>
<td></td>
</tr>
<tr>
<td>\textit{Bam}HI F primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>TCCCACTGTATTACGACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GGCAATGGGAGCTTTGTAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RE = Restriction enzyme.
in table 1. Totally 192 EBV-positive cases, consisting of 124 NPC cases and 68 TW cases from healthy donors, were included in the study. All the subjects were inhabitants of the province of Shandong with local dialects, and they all gave informed consent for the study.

**Cell Controls**

The cell lines of B95-8, P3HR1 and Raji were used as positive controls, for the EBV types A, B and D, respectively. In addition, B95-8 was also used as a positive control of the EBV type F. EBV-negative Ramos cell served as a negative control.

**DNA Extraction**

DNA extraction from the control cell lines and TWs was carried out using the standard method with proteinase K digestion and phenol-chloroform purification. QIAamp® DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) was used to extract the DNA from paraffin-embedded NPC tissues.

**Polymerase Chain Reaction Amplification**

The EBV BamHI W fragment, EBNA3C gene, BamHI F region and BamHI W1/I1 region were amplified by the polymerase chain reaction (PCR) technique. The primer sequences and the size of PCR products are shown in table 1. PCR was performed with 2 μl of DNA extracts (100 ng/μl) in a 25-μl reaction mixture containing standard PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP, 0.5 μM of each primer and 1.25 U Taq DNA polymerase. The DNA amplification protocol included 1 cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The program ended with 10 min at 72°C. The reaction was carried out in GeneAmp PCR system 2700 (Applied Biosystems, Foster City, Calif., USA). PCR products were analyzed by using electrophoresis in a 2% agarose gel. After electrophoresis, the gels were stained with ethidium bromide and photographed under the UV light transilluminator. The representative PCR products were analyzed using an ABI 3730 DNA sequencer to confirm genotype identity.

**Enzymatic Digestion of PCR Products**

The type C/D and type F/f are based on the BamHI digestion of each PCR product. The products of the PCR were purified using a gel extraction kit (Qiaex II; Qiagen GmbH) according to the instructions of the manufacturer. The enzymatic reactions were performed in a 20-μl reaction mixture containing 10 μl of PCR products, 1× reaction buffer and 10 units of BamHI. After incubation at 30°C for 4 h, the DNA products were analyzed on 2% agarose gel, and then visualized by ethidium bromide staining.

**Results**

**Analysis of EBNA3C Gene (A/B Types)**

The expected amplification products were 153 bp for EBV type A and 246 bp for type B (fig. 1). Of the 124 EBV-associated NPC samples, 99 (79.8%) harbored EBV type A, and 25 (20.2%) carried type B. The EBNA3C region was not amplified in 9 of 68 healthy donors (table 2). Among 59 healthy TWs with amplification of the EBNA3C region, 45 (76.3%) were positive for the type A strain alone, 12 (20.3%) for type B, and 2 (3.4%) for both types. The differences in the distribution of types A and B between the NPC patients and the healthy donors were not statistically significant (p > 0.05).
The determination of the EBV types C or D was based on the presence of an additional BamHI enzyme site at the BamHI W1/I1 region. Cleavage with the BamHI restriction enzyme yields 2 fragments of 130 and 76 bp for type D, while 206 bp for type C (fig. 2). The representative PCR products were analyzed by a DNA sequencer and the analysis of the representative sequence is shown in figure 3. The BamHI W1/I1 region was not amplified in 3 of 124 (2.4%) NPC samples and in 16 of 68 (23.5%) healthy TWs. Of the 121 NPC samples, the EBV genotype C was confirmed in 82 (67.8%) cases, and genotype D was confirmed in 39 (32.2%) cases. Among the 52 healthy donors, genotypes C and D were found in 36 (69.2%) and 16 (30.8%), respectively (table 2). The frequency of EBV genotypes C and D showed no significant differences between the NPC patients and healthy donors (p > 0.05).

**Analysis of BamHI F Region (F/f Types)**

Type F or f at the BamHI F region were also determined by the digestion with the BamHI restriction enzyme after PCR amplification. The product size 198 bp was interpreted as type F, and 2 fragments of 127 and 71 bp were recognized as type f (fig. 4). No BamHI F DNA fragment was detected in 4 of 124 NPC and 1 of 68 healthy TW samples. Of the 120 NPC specimens, 105 (87.5%) harbored EBV type F and 15 (12.5%) carried type f. Among the 67 healthy donors, 64 (95.5%) cases were classified as type F and 3 (4.5%) cases were as type f (table 2). The distribution of type F and type f in both distinct population groups was not significant (p > 0.05). The analysis of the representative sequence is demonstrated in figure 5.

**Comprehensive Analysis of EBV Genotyping in NPC Patients and Healthy Controls**

We analyzed the copresent frequency of the polymorphisms at the BamHI F region, BamHI W1/I1 boundary region and EBV subtypes in 117 NPC samples and 47 TW samples. The predominant combination of EBV geno-
types was types A, C or F. This combination was confirmed in 65 out of 117 (55.6%) EBV-associated NPC patients as well as in 32 of 47 (68.1%) healthy donors. There were no significant differences in this dominant combination of EBV genotypes between the NPC patients and healthy donors (p > 0.05).

Discussion

In the present study, we characterized EBV polymorphisms at the EBNA3C gene (type A or B), BamHI W1/I1 region (type C or D) and BamHI F region (type F or f) in NPC patients and healthy individuals in Northern China. To our knowledge, this is the largest study of EBV polymorphisms on viral strains from Chinese cases in a nonendemic area of NPC. It has been reported that EBV type A is predominant throughout the world except for equatorial Africa, Papua New Guinea and Alaskan Eskimos [3, 18, 19]. As observed in most regions in the world, our study shows that EBV type A is predominant in both NPC patients and healthy individuals, which is consistent with a previous study by Zhou et al. [20], who reported that EBV type A was detected in 11/13 (84.6%) healthy TWs and 65/71 (91.5%) patients with Hodgkin’s disease from Northern China. A high prevalence of EBV type A was identified in 24/25 NPC patients from Guangdong, 37/45 NPC patients from Guangxi and 25/30 NPC patients from Taiwan, all belonging to the Southern China, endemic area of NPC [18, 21, 22]. Type A is also the prevalent strain in human malignancies in other Asian countries, such as Malaysia, Japan, and Korea [11, 23, 24]. Generally, type B was reported to prevail in Africa and

Fig. 4. Restriction fragment length polymorphism analysis with BamHI restriction enzyme digestion after PCR amplification at the BamHI F region. The digestion results in 2 bands of 127 and 71 bp indicating the presence of the BamHI site (type f). Lane M = DL2000 DNA marker; lane 1 = positive control of type F (cell line B95-8); lanes 2, 3, 6 and 7 = representative cases of the type F strains; lanes 4 and 5 = representative cases of type f strains.

Fig. 5. The EBV genotype F and f sequence analysis of the representative PCR products. a Representative NPC samples (type F) which lack the BamHI restriction site. b Representative NPC samples (type f) which possess the BamHI site in the corresponding region. The arrow indicates the BamHI restriction site.
Alaskan Eskimos [3, 19]. However, Sixbey et al. [25] and Apolloni and Sculley [26] also reported that type B can be detected in 41% (14/34) of healthy donors from the United States and 33% (7/21) of healthy donors from Australia, suggesting that type B virus may also be widespread in nature.

Although biological differences attributable to EBV types A and B provide a rationale for the classification of EBV into these 2 broad groups, these differences do not fully account for the natural diversity of EBV. We also identified a type C strain as predominant in Northern China. This result is similar to that of previous studies on Asian populations but different from those on Western cases. Lung et al. [9] reported a much higher prevalence of type C both in NPC biopsies and TWs of healthy donors from Southern China. Tamura et al. [11] and Sidagis et al. [14] also detected the presence of type C in 7/10 (70%) NPC tissues and 50/54 (92.6%) TWs of healthy donors in Japan. Contrary to most districts of Asia, type D was found to be common in NPC tissues from Europe and North Africa where there is an intermediate incidence of NPC [8, 10]. In Slovenia and Tunisia, type D strain was found in 30/48 and 46/47 NPC biopsies, respectively [10, 27]. In addition, the presence of the type D virus was also found in 10/12 (83.3%) Caucasian NPC patients and in 29/36 (81%) healthy donors from the United States [13, 28]. From the above data, we concur with the concept that distinct EBV strains predominate in different geographical areas [3]. Interestingly, the incidence of type C in NPC patients (67.8%, 82/121) and healthy individuals (69.2%, 36/52) from Northern China is lower than that in Southern China, where 28/28 (100%) NPC biopsies and 7/7 (100%) TWs of healthy donors carried this variant [9]. The difference was statistically significant (p < 0.05). One explanation of the difference may be due to the limited number of samples from Southern Chinese people which were analyzed. Full-scale molecular epidemiological studies to map the distribution of type C/D from Southern and Northern China are needed to get a clear-cut explanation for the difference.

The other variant of particular significance, designated type F variant, has been suggested to be associated with NPC in endemic areas [9, 29]. Lung et al. [9, 29] reported that the F variant had a higher frequency in NPC patients (74.0%, 57/77) as compared to healthy individuals and patients with other tumors (7.7%, 1/13) or patients who are in remission for NPC for over 3 years (20.9%, 14/67) in Southern China. Based on these findings, they deduced that type F of EBV may be associated with NPC. A high frequency of type F was also detected in NPC patients from Taiwan (50.0%, 15/30) and Guangxi (79.2%, 19/24), areas which also belong to the endemic region of NPC in Southern China [21, 22]. However, our study showed that type F was found in only 12.5% (15/120) of NPC patients and 4.5% (3/67) of healthy individuals, respectively. The frequency of type F in NPC patients from Northern China is significantly lower than that in the NPC endemic area (p < 0.05). Similar to our results, the F variant was rare in other areas, such as Japan, Korea, Europe, North America, South America and Africa ignoring the source (healthy donors and malignant patients) [10, 11, 24, 27, 30, 31]. In addition, the F variant was dominant in patients with other EBV-associated diseases (18/19 T cell lymphoma, 3/3 infectious mononucleosis and 37/45 EBV-associated gastric carcinoma) in Southern China [22], whereas the f variant was only prevalent in NPC of Southern China. However, the small number in the control group may limit the conclusion that the f variant is associated with the development of NPC. Larger-scale molecular epidemiological studies from the endemic region are needed to clarify this issue.

In addition, we found no evidence of special EBV genotypes associated with NPC in Northern China since the genotyping A/B, C/D and F/f of the EBV variant detected in NPC patients occurred with similar frequency in healthy virus carriers from the same geographical region. This finding is in line with the other previous reports. In the UK and Southern Japan, the prevalent strains in EBV-related malignancies were the same as in the healthy population in the same geographical region [14, 32]. In summary, types A, C or F are the most common subtypes in NPC patients and healthy donors in Northern China. The distribution of genotypes as well as the prevalence of the major combination genotype ACF in both NPC patients and healthy individuals are similar. Therefore, it appears that distributions of the EBV genotypes are geographically restricted rather than NPC restricted.

Whether the specific EBV subtypes preferentially associate with particular malignancies or represent geographical polymorphisms remains unresolved. Given the ubiquitous EBV infection in the human population, factors other than EBV also contribute to the etiology of NPC. Host genetics has been suggested to be associated with NPC risk [33–36]. Several studies showed that NPC risk was significantly higher in first-degree relatives of cases than in the general population [37–39]. The finding of up to 50% incidence of type F in NPC biopsies from first- and second-generation immigrant Chinese people suggests that a particular genotype can persist through
several generations [13]. In addition, environmental risk factors such as salted fish consumption, high preserved vegetables consumption during childhood, occupational exposures to wood and betel nut use might be associated with NPC [40]. The etiology of NPC is multifactorial, and the exact mechanisms certainly deserve to be explored further.

Acknowledgments

This research was supported by a grant from the National Natural Science Foundation of China (NSFC 30740068 and NSFC 30970157) and the National Science Foundation of Shandong Province (Y2008C90). The authors thank Marshall Austin, MD/PhD (Professor of University of Pittsburgh), for critical review of the manuscript.

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