Nasal natural killer/T-cell lymphoma and its association with type ‘‘i’’/XhoI loss strain Epstein–Barr virus in Chile

M E Cabrera, Y Eizuru, T Itoh, C Koriyama, Y Tashiro, S Ding, S Rey, S Akiba, A Corvalan

Background: Nasal T/natural killer (NK)-cell lymphoma is an aggressive type of non-Hodgkin’s lymphoma associated with Epstein–Barr virus (EBV) and striking geographical variations worldwide.

Aim: To characterise nasal NK/T-cell lymphoma associated with genotypes of EBV in Chile, a Latin American country, where multiple strains of EBV, including two new recombinant strains, in healthy individuals were recently found.

Methods: Cases with diagnosis of primary nasal lymphoma were selected for histological and immunohistochemical analysis (CD3, CD3e, CD4, CD8, CD79a, CD56, CD57 and TIA-1) and in-situ hybridisation, serology and genotyping analysis for EBV.

Results: Out of 22 cases, 9 (41%) cases fulfilled the World Health Organization criteria for nasal NK/T-cell lymphoma; of these 7 (78%) cases were positive for EBV. Genotyping analysis revealed 6 cases of type 1 EBV and wildtype F at the BamHI-F region, 4 cases type ‘‘i’’ EBV at the BamHI-W1/W11 region; XhoI wild type was found in 2 and XhoI loss in 4 cases, respectively. Co-segregation analysis of the BamHI-W1/W11 region and XhoI restriction site showed the new recombinant strain type ‘‘i’’/XhoI loss in 3 cases and type ‘‘i’’/XhoI wild-type strain in 1 case. Most patients were treated with combined antirachycine-containing regimens. Half of the cases attained complete remission.

Conclusion: Although nasal NK/T-cell lymphomas from Chile share similar clinicopathological features, high association with EBV and unfavourable prognosis with those described elsewhere, genotype analysis shows that the new recombinant type ‘‘i’’/XhoI loss strain might contribute to explain the intermediate incidence of nasal NK/T-cell lymphomas in Latin America.
WHO Classification for extranodal NK/T-cell lymphoma, nasal type, which is the subject of this study.

**Histology and immunohistochemistry**

Paraffin-wax-embedded sections were stained with H&E, and all cases were classified according to the new WHO Classification. Immunohistochemical analysis was conducted using the following monoclonal antibodies: CD3 (Novocastra Laboratories, Newcastle upon Tyne, UK, dilution 1:100); CD3e (Dako, Glostrup, Denmark, dilution 1:50); CD4 (Novocastra Laboratories, dilution 1:40); CD8 (Dako, Denmark, dilution 1:25); CD79a (Dako, Copenhagen, Denmark, dilution 1:25); CD56 (Novocastra Laboratories, dilution 1:100); CD57 (Immunotech, Marseille, France, dilution 1:1); and TIA-1 (Coulter Immunology, Hialeah, Florida, USA, dilution 1:2). The avidin–biotin-peroxidase complex method was used (VECTASTAIN ABC KIT, Vector Laboratories, Burlingame, California, USA). The expression of each molecule was graded as follows: (1) \(-\), (2) + (1–49%) and (3) ++ (>50%), according to the proportion of positive cells. Replicate slides devoid of monoclonal antibodies were included as a negative control.

**In-situ hybridisation for EBV-encoded small RNA type 1**

EBV-encoded small RNA type-1 (EBER-1) expression was detected with a complementary digoxigenin-labelled 30-base oligomer, using the procedure described previously. A case was considered to be EBER-1 positive based on a positive signal under microscopy in at least 10% of tumour cells. Lymph node section from a patient with infectious mononucleosis was used as positive control, and a sense probe for EBER-1 was used as negative control.

**Genotype-specific primer sets and probes for EBV**

Genotypes of EBV were examined by PCR, restriction enzyme and Southern blot analysis as described previously. A case was considered to be type 1 positive based on a positive signal under microscopy in at least 10% of tumour cells. Lymph node section from a patient with infectious mononucleosis was used as positive control, and a sense probe for EBER-1 was used as negative control.

**Table 1. Clinical features of nasal natural killer/T-cell lymphomas**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Tumour involvement</th>
<th>Clinical stage</th>
<th>Treatment</th>
<th>Response</th>
<th>Survival period, months</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62/F</td>
<td>Nose</td>
<td>I</td>
<td>CT</td>
<td>Failure</td>
<td>5</td>
<td>Died</td>
</tr>
<tr>
<td>2</td>
<td>24/F</td>
<td>Nose</td>
<td>I</td>
<td>CT and RT</td>
<td>Failure</td>
<td>4</td>
<td>Died</td>
</tr>
<tr>
<td>3</td>
<td>52/F</td>
<td>Nose</td>
<td>I</td>
<td>CT</td>
<td>Failure</td>
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<td>Alive</td>
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<tr>
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<td>40/M</td>
<td>Nose</td>
<td>I</td>
<td>CT</td>
<td>CR</td>
<td>12</td>
<td>Alive</td>
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<tr>
<td>5</td>
<td>28/M</td>
<td>Nasopharynx, supraglottis, stomach</td>
<td>IV</td>
<td>CT</td>
<td>Failure</td>
<td>1</td>
<td>Died</td>
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<tr>
<td>6</td>
<td>58/F</td>
<td>Nose</td>
<td>I</td>
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<td>NA</td>
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<td>NA</td>
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<tr>
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<td>Relapsed</td>
</tr>
<tr>
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<td>43/M</td>
<td>Hard palate, orbit, ethmoid</td>
<td>II</td>
<td>CT</td>
<td>CR</td>
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<tr>
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CR, complete remission; CT, chemotherapy; F, female; M, male; NA, not available; RT, radiotherapy.

**Serology for human T-lymphotropic leukaemia virus type-1 and HIV**

We conducted serological analyses of human T-lymphotropic leukaemia virus type-1 (HTLV-1) and HIV by Enzyme Immunoassay (Abbott, Abbott Park, Illinois, USA).

**Treatment**

The treatment for the cases was anthracycline-contained CH, such as cyclophosphamide, doxorubicin hydrochloride, oncovin and prednisolone or cyclophosphamide, doxorubicin hydrochloride, oncovin, prednisolone and bleomycin, or a combination therapy with RT at a dose of 40–50 Gy.

**RESULTS**

**Patients’ characteristics**

Table 1 summarises clinical data obtained from nine patients with extranodal nasal type lymphoma. There were five women and four men. Their median age was 43 years (range 24–62). Family names suggested that all nine patients were of Hispanic origin. The nasal cavity was the main site involved in all patients, but one, which presented with a middle-line mass of the hard palate and invasion to adjoining tissues such as the nasopharynx, ethmoid and orbit. In all, 8 (88%) patients had a localised disease (stage I–II), except one (stage IV) with stomach involvement.

**Histology and immunohistochemistry**

There was a broad cytologic spectrum of tumour cells, from atypical small cells, to a mixture of small and large cells to large cells, with frequent mitotic figures, admixing with a variable number of inflammatory cells, such as granulocytes, macrophages, plasma cells and small lymphocytes. Angiocentricity and angioinvasive pattern were reported in all cases. Necrosis was also found in all cases, but was most prominent in tissues with large cells. Table 2 summarises the immunophenotypic findings. Six patients met the criteria of the WHO Classification for nasal lymphoma (CD3e positive, CD56 positive, TIA-1 positive and CD79a negative). Three patients were CD3e positive, TIA-1 positive, CD79a negative but CD56 negative.

**Serology to HTLV-1 and HIV**

Antibodies against HTLV-1 and HIV were negative in four patients studied.

**EBV in-situ hybridisation and genotyping analysis**

In total, 7 (78%) cases were considered EBV positive according to the expression of EBER-1 by in-situ hybridisation in \(\geq 10\%\) of the tumour cells (table 2). Two cases were negative for EBER-1 expression. Figure 1 shows the representative examples of EBV amplified by primer pair. Type I, 205 bp fragment, and type et al et al., and XhoI restriction enzyme resulted in 67 and 46 bp product for type 1, prototype F, type I and XhoI wildtype virus. Cloned BamHI-“i” DNA fragments served as positive controls for variant “f” and type “i”, respectively.

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<td>Died</td>
</tr>
<tr>
<td>6</td>
<td>58/F</td>
<td>Nose</td>
<td>I</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</tr>
<tr>
<td>7</td>
<td>53/F</td>
<td>Nose (at 6 months)</td>
<td>I</td>
<td>CT and RT</td>
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<td>Nose</td>
<td>I</td>
<td>CT</td>
<td>CR</td>
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</tr>
</tbody>
</table>

CR, complete remission; CT, chemotherapy; F, female; M, male; NA, not available; RT, radiotherapy.
positive nasal lymphoma cases. Genotyping analysis revealed that six out of seven positive cases were type 1 EBV and wild-type F at the BamHI-F region. The remaining case (case 3) did not amplify for type 1 or 2 EBV, and was variant “i” at the BamHI-F region. All four amplified cases of the BamHI-W1/I1 region were type “i” EBV. In the remaining three cases, no amplification of this region was successful in six cases, two were XhoI wild type and four showed XhoI loss. Figure 2 shows the representative examples. As it is known that polymorphisms at the BamHI-W1/I1 region cosegregate with XhoI restriction site polymorphisms, we analysed these two variants. Among four type “i” nasal lymphomas, three harboured a novel recombinant strain type “i’/XhoI loss (cases 4, 7 and 8) and one harboured type “i’/XhoI wildtype strain (case 9).

### DISCUSSION

The present study shows that the clinical and phenotypic characteristics of nasal NK/T-cell lymphoma in Chile are similar to those described in other Latin American as well as Asian and Western countries. Here, we also confirm that most nasal NK/T-cell lymphomas were EBV positive,1 although we failed to show this association in two cases. Possible explanations may be the low quality of preservation of paraffin-wax-embedded blocks, or the lack of EBER-1 expression, as has been shown in other tumours.

Nevertheless, these negative cases strictly met the criteria for classification as nasal NK/T-cell lymphomas, as they were CD56 positive, CD3ε positive and TIA-1 positive. Genotype analysis showed the presence of a new recombinant strain characterised by the presence of an extra fragment (case 3). The second fragment after BamHI digestion (46 bp) failed to show this association in two cases. Possible explanations may be the low quality of preservation of paraffin-wax-embedded blocks, or the lack of EBER-1 expression, as has been shown in other tumours.1 Nevertheless, these negative cases strictly met the criteria for classification as nasal NK/T-cell lymphomas, as they were CD56 positive, CD3ε positive and TIA-1 positive. Genotype analysis showed the presence of a new recombinant strain characterised by the presence of an extra fragment (case 3).

### Table 2

Summary of immunohistochemical and Epstein–Barr virus RNA in-situ hybridisation results in nasal natural killer/T-cell lymphomas

<table>
<thead>
<tr>
<th>Case</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD79a</th>
<th>CD56</th>
<th>CD57</th>
<th>CD3e</th>
<th>TIA-1</th>
<th>EBER (%)</th>
<th>Type</th>
<th>F/“i”</th>
<th>I/“i”</th>
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<td>++</td>
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<td>“i”</td>
<td>Loss</td>
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<td>80</td>
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<td>NA</td>
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<tr>
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<td>−/−</td>
<td>−/−</td>
<td>++</td>
<td>70&lt;</td>
<td>1</td>
<td>F</td>
<td>“i”</td>
<td>Loss</td>
</tr>
<tr>
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<td>−/−</td>
<td>−/−</td>
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<td>−/−</td>
<td>−/−</td>
<td>++</td>
<td>70&lt;</td>
<td>1</td>
<td>F</td>
<td>“i”</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>

EBER, Epstein–Barr virus-encoded small RNAs; NA, not amplified; NT, not tested.

*In-situ hybridisation results were expressed as number of positive cells per medium-powered field (>200). Cases were considered positive when a positive signal was observed in ≥10% of the tumour cells.

1. Background was also reacted.

2. Cytoplasmic expression was observed.

3. Positive for reactive cells but not for neoplastic cells.

### Figure 1

Identification of Epstein–Barr virus (EBV) in a case of nasal natural killer/T-cell lymphoma (case 1) by EBV-encoded small RNA type-1 in situ hybridisation (×400).

Figure 2

Genotypes of Epstein–Barr virus in cases of nasal natural killer/T-cell lymphomas. (A) Southern blot analysis after PCR amplification and hybridisation with specific probes for type 1 and 2 strains. (B) Southern blot analysis after PCR amplification, digestion with BamHI restriction enzyme and hybridisation with specific probes for polymorphisms at the BamHI-F region. (C) Polyacrylamide gel after PCR amplification and digestion with BamHI restriction enzyme at the BamHI-W1/I1 region. (D) Polyacrylamide gel after PCR amplification and digestion with XhoI restriction enzyme at XhoI site polymorphisms. The second fragment after XhoI digestion (46 bp fragment) for the wild type is not seen in the gel.
Take-home messages

- Six out of seven Epstein-Barr virus (EBV)-positive nasal natural killer (NK)/T-cell lymphomas were type 1 EBV and wildtype F at the BamHI-F region.
- Among six EBV-positive nasal NK/T-cell lymphomas, four were type "i" EBV at the BamHI-W1/I1 region.
- Among six EBV-positive nasal NK/T-cell lymphoma cases, four showed XhoI loss and two had XhoI wildtype restriction site.
- Case segregation analysis of the BamHI W1/I1 region and XhoI restriction site showed a novel recombinant strain type "i"/XhoI loss in three cases and type "i"/XhoI wildtype strain in one case.
- Genotype analysis revealed that the novel recombinant strain type "i"/XhoI loss might contribute to explain the intermediate incidence of nasal NK/T-cell lymphomas in Latin America.

BamHI site at the BamHI-W1/I1 region, together with the loss of the XhoI restriction site at the LMP1 gene (type "i"/XhoI loss strain). Type "i"/XhoI loss strain has been recently identified in a proportion of 329 healthy adults in two Latin American countries, including Chile.26 In that study, type "i"/XhoI loss strain was found admixed with another recombinant strain, type 1/XhoI wild-type strain, and strains described previously in Western countries (type "i"/XhoI wild-type strain) and Asian countries (type 1/XhoI loss).26-28 Therefore, these results provide the first evidence that type "i"/XhoI loss strain can be found in nasal NK/T-cell lymphomas. Furthermore, the presence of type "i"/XhoI loss might contribute to explain the intermediate incidence of nasal NK/T-cell lymphoma in Latin America, in between that in Western and in Asian regions.16-18 The frequency of other genotypes, type 1 and wild-type F at the BamHI-F region in our patients were similar to that of patients with nasal NK/T-cell lymphoma described in Japan.26

Even early stages have an overall survival rate lower than that in other extranodal or nodal lymphomas. Besides being a resistant disease, relapses are also frequent.27 To date, there is no agreement on the best treatment. RT is effective in limited resistant disease, relapses are also frequent.36 To date, there is no agreement on the best treatment. RT is effective in limited disease but does not ensure prolonged survival.56 Recent reports suggested that high dose CH with stem-cell transplantation may be an effective treatment modality for refractory NK lymphomas.37 The survival benefit of this modality still needs to be shown.

In conclusion, we describe nasal NK/T-cell lymphomas from Chile, with similar clinicopathological features as those described elsewhere, strong association with EBV and unfavourable prognosis. Genotype analysis revealed the presence of a novel recombinant strain (type "i"/XhoI loss) in most of the analysed cases, providing evidence that, although previously described in healthy donors, this strain can also be found in nasal NK/T-cell lymphomas. Further research is necessary to clarify whether this finding is specific of Latin America or can be found in Western and Asian countries as well.

ACKNOWLEDGEMENTS

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