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Epstein-Barr virus infection in sinonasal non-Hodgkin's lymphomas

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Abstract Sinonasal non-Hodgkin's lymphomas (SNHLs) of B- or T-cell immunophenotype have been associated with Epstein-Barr virus (EBV) infection of neoplastic lymphoid tissue. Nine SNHLs were investigated using immunohistochemistry, the polymerase chain reaction (PCR) for EBV genome and in situ hybridization (ISH) for EBV encoded RNAs (EBER), immunoglobulin (CIgHR) and clonal T-cell receptor (CTCβR) gene rearrangements. Eight cases were diagnosed as peripheral pleomorphic T-cell lymphomas (pPTCL). PCR showed the presence of EBV genome in eight cases; ISH for EBER led to the detection of positive cells in five cases. Late membrane protein (LMP) immunostaining was observed in three cases. No EBV positivity has been detected in control cases. The frequent association with EBV infection in the cases illustrated confirms the previous suggestions that EBV may have a role in the genesis of lymphomas of the sinonasal region.

Key words Sinonasal non-Hodgkin's lymphomas
Epstein-Barr virus infection · Polymerase chain reaction
In situ hybridization · Late membrane protein

Introduction

Sinonasal non-Hodgkin's lymphomas (SNHLs) constitute 0.44–2.2% of all extranodal lymphomas and 6.4–13% of extranodal lymphomas of the head and neck [4, 7, 9, 10, 32]. They are the most frequent non-epithelial malignancy of the nose and paranasal sinuses [2, 4, 7, 8, 10, 12, 14, 16, 28, 31]. A T-cell immunophenotype predominates in Far East and Caucasian populations [5, 14, 16, 22, 24, 27, 28]. In Western countries, some authors have reported a prevalence of B-cell lymphomas [7, 11, 31], others of T-cell lymphomas [2, 6, 8, 23], or a similar percentage of B- and T-cell lymphomas [4].

Recently, EBV genome has been identified in SNHLs, mostly of T-cell immunophenotype, suggesting its possible role in lymphomagenesis [2, 14, 16, 20, 31].

In the present study, nine cases of primary SNHL have been analysed by means of immunohistochemistry, polymerase chain reaction (PCR) and in situ hybridization (ISH) for EBV genome and EBV encoded RNAs (EBER), immunoglobulin (CIgHR) and T-cell receptor (CTCβR) gene rearrangement.

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Materials and methods

Nine cases of primary SNHL have been diagnosed at the Institute of Pathological Anatomy and Histology of the University of Siena in the period 1983–1993. They were classified according to the updated Kiel classification [26] and staged according to the American Joint Committee Staging System for nasopharyngeal primary tumours [1].

Twenty nasal biopsies with inflammatory lesions were used as controls.

Tissues from cases and controls were fixed in formalin and embedded in paraffin, and sections were routinely stained.

Immunophenotyping on paraffin sections was performed using the alkaline phosphatase anti-alkaline phosphatase method. The monoclonal antibodies used were:L 26 (CD20,L26,Dako); 4KB5 (CD45RA, Dako); UCHL1 (CD45RO,Dako); BF1 (T-cell Diagnostics); CD3 (CD3, T3-4B5, Dako); MT1 (CD43, DF-T1, Dako); LMP (EBV,CS1-4, Dako); BCL2 (BCL2,124, Dako). Normal human tonsils were used as positive controls. For negative controls, we replaced the primary antibody with normal mouse serum.

For the PCR 10 µ thick tissue sections were deparaffinised, washed in 100% ethanol, dried, and incubated for 3 h at 55° C in 100 µl of digestion buffer (50 mM TRIS-HCl, pH 8.5; 1 mM EDTA; 0.5% Tween 80) containing 200 µg/ml of proteinase K (Boehringer, Mannheim, Germany). After boiling for 15 m, 10 µl of the material were used for amplification (template DNA solution). The primers were synthetized using DNA synthetizer (M-Medical, Florence, Italy). Amplification was performed on a DNA thermal cycler (Violet, Biostar, Rome, Italy).

The primers for EBV corresponded to sequences localized at 14695–14719 and 14825–14849 of the EBV genome (EBV 1:5′-GAGGTCAGGTTACTTACCCCTGAAG-3′; EBV2: 5′TCTCAGGGTCCCCT-CGGACAGCTCC-3′). Aliquots of 10 µl DNA were made up to 100 µl containing 50 mM potassium chloride,

1.5 mM magnesium chloride, 10 mM TRIS-HCl (pH 8.8), 0.1% Triton X-100, 0.2 mM each dATP, dCTP, dGTP, dTTP, 0.1 nM of each primer, 2 units *taq* polymerase (Promega, Madison, Wi., USA) and overlaid with one small drop of mineral oil. Amplification was programmed for 40 cycles of 30 s denaturation at 95° C, 30 s annealing at 55° C and 90 s extension at 72° C; for the last cycle the extension was 5 min at 72° C. This primer pair gives rise to 155 base-pair (bp) amplified products. The positive controls used were the Namalwa cell line harboring 1 to 2 copies of EBV and EBV-positive case of Hodgkin's disease. The negative controls were the EBV-negative cell line HUT102 and DNA-negative samples.

PCR to amplify portions of the rearranged T-cell antigen receptor B-chain was carried out according to the procedure described by McCarthy et al. [18] as slightly modified in our laboratory. Primers combination was restricted to DB2 (5′-TCATGGTGTAACATTGTGGGGAC-3′) and JB2 (5′-AGC-AC(TCG)GTGAGCC(TG)GGT-GCC-3′) sequences because preliminary studies in our and other laboratory [25] showed that using a large repertoire of primers did not significantly increase the number of positive detected. The reaction mixture consisted of aliquots of 5 µl di DNA made up to 50 µl containing 50 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM TRIS-HCl (pH 8.8), 0.1% Triton x-100, 0.25 mM each dATP, dCTP, dGTP, dTTP, 30 ng of each primer, 2.5 units *Taq* polymerase (Promega) and overlaid with one small drop mineral oil. Amplification was programmed for 36 cycles of 30 s denaturation at 95° C, 40 s annealing at 52° C, 30 s extension at 71° C; for the last cycle the annealing was 10 min at 52° C and the extention 2 min at 71° C. This primer pair gives rise to 70–100 bp amplified products. Negative control for reagent contamination, consisting of all PCR reagents except template DNA, were applied each time a PCR assay was performed. DNA extracted from a peripheral T-cell lymphoma was employed as positive control.

For detecting monoclonality at the DNA level in B lymphocyte population we applied a slightly modified version of the method described by Wan et al. [29], which allows amplification of the V-D-J region of the heavy chain gene. A semi-nested PCR was carried out using the primers Fr3A (5′-ACACGGC(CT)(GC)TGTATTACTGT-3′), LJH (5′-TGAGGA-GACGGTGACC-3′) and VLJH (5′-GTGACCAGGGT(AGCT)-CCTTGGCCCCAG-3′). The reaction mixture (50 µl) contained 10 µl of the template DNA solution; 0.25 mM each of dATP, dGTP, dTTP, dCTP; 0.05 µg of each primer; 2.5 units of *taq* polymerase (Promega); and buffer (Promega) with 4.5 mM magnesium chloride. Each reaction mixture was overlaid with a small drop of mineral oil. PCR was performed: a first round of 40 cycles with the primers Fr3A and LJH and a second round of 30 cycles with Fr3A and VLJH using 10 µl of the 1:1000 diluted first PCR product as a template. A PCR cycle consisted of denaturation for 60 s at 95° C, annealing for 50 s at 56° C and extension for 60 s at 72° C. A discrete band (100–120 base pair long) indicates monoclonality. Negative controls for reagent contamination, consisting of all PCR reagent except template DNA, were applied each time a PCR was performed. DNA extracted from a large cell follicular centre cell lymphoma was employed as positive control.

The PCR product (10–20 µl) was examined in a 2% agarose gel and stained with ethidium-bromide for the presence of appropriate bands. The amplification of a section of the β globin gene (Perkins Elmer, Chicago, USA) served to determine the amplifiability of extracted DNA.

For ISH 5 µ thick sections were mounted and dewaxed. 200 µl of proteinase K (Boehringer, Mannheim, FRG), 3 µg/µl in 50 mM TRIS HCl, pH 7.6, were added for 30 min at 37° C. The slides were washed in pure water, dehydrated in ethanol, air-dried and then incubated with 30 µl of EBV-oligonucleotides/fluorescein isothiocyanate (FITC) (complementary to the two nuclear EBER RNAs encoded by EBV (Dako) for 2 h at 37° C. They were washed and incubated with 150 µl of rabbit F(ab′) anti FITC/AP for 30 min. After being washed and buffered, they were incubated with 10 ml of substrate solution containing 32 µl of 5-bromo-4chloro-3-indolyl-phosphate (BCIP) (50 mg/ml) and 64 µl of nitroblue tetrazolium (50 mg/ml) for 2 h at 37° C, and finally counterstained with fuchsine. EBV-positive sections from cases of Hodgkin's disease served as positive controls, while EBV-negative lymphoid tissue served as negative control.

Any slides negative for EBV DNA and all non-neoplastic control cases were tested for viability of total RNA using a poly d(T) probe.

Results

The clinical data are illustrated in Table 1. The histological picture was characterized by a polymorphic lymphoid infiltrate composed of a mixture of small lymphocytes and variable numbers of atypical lymphoid cells and immunoblasts along with reactive plasma cells, occasionally eosinophils and histiocytes. Scattered Reed-Sternberg-like cells were observed in three cases.

Immunophenotyping and immunogenotyping are summarized in Table 2. Immunohistochemistry showed a T cell phenotype in eight cases. One case (case 7) gave inconclusive results probably due to inadequate fixation. Few reactive B-lymphocytes were intermingled with neoplastic T-cells. T-cell clonality at the DNA level was observed in five cases by the presence of a 70–100 base pair (bp) band indicative of TCRβ chain rearrangement. IgH gene rearrangement was not detected in any case.

All the cases were diagnosed as peripheral pleomorphic T-cell lymphomas (pPTCL) with the exception of case 7, in which the cell lineage could not be established.

Polymerase chain reaction for EBV showed a 155 bp amplified DNA in eight cases (Fig. 1). ISH for EBER led to the detection of positive cells in five cases out of eight PCR positive cases. In two cases almost all the atypical lymphoid cells (more than 80%) showed a strong signal. In the other cases only a variable numbers of tumour cells were positive. The small cells were usually negative although by morphological criteria they belonged to the neoplastic population (Fig. 2). LMP immunostaining was

Table 1 Clinical data of nine sinonasal non-Hodgkin's lymphomas

Case no	Age (year)/sex	Presenting symptoms	Tumour involvement	Clinical stage[a]
1	31/F	Nasal obstruction, purulent discharge	Nose	T2
2	48/M	Nasal mass	Nose	T3
3	53/M	Nasal mass, rhinorrhea	Nose, maxillary sinus	T2
4	40/F	Nasal obstruction, epistaxis, epiphora	Nose, frontal sinus	T2
5	52/M	Nasal obstruction	Nose, maxillary sinus	T3
6	38/M	Nasal mass	Nose	T2
7	83/M	Nasal obstruction, epistaxis	Nose	T2
8	60/M	Nasal obstruction, anosmia	Nose, maxillary sinus	T3
9	27/M	Nasal mass	Nose	T3

[a] According to American Joint Committee Staging System (1988)

Table 2 Summary of histological, immunohistochemical, immunogenotypic, polymerase chain reaction and in situ hybridisation findings in sinonasal non-Hodgkin's lymphomas. (*pPTCL*, peripheral pleomorphic T-cell lymphoma; *PCR*, polymerase chain reaction; *UN*, malignant lymphomas, unclassified; *ISH*, in situ hybridisation; *LMP*, latent membrane protein, demonstrated by immunohistochemistry; *EBV*, Epstein-Barr virus DNA; *PCR*, polymerase chain reaction; *EBER*, in situ hybridisation for EBV-encoded RNAs; *CIgHR*, clonal immunoglobulin heavy chain gene rearrangement; *CTCRβR*, clonal T-cell receptor *β*-rearrangement)

Case no	Histology	CD20	UCHL1	CD3	βF1	BCL2	LMP	CIgHR (PCR)	CTCRβR (PCR)	EBV (PCR)	EBER (ISH)
1	pPTCL	–	+	+	+	–	–	–	–	+	–
2	pPTCL	–	+	+	+	–	–	–	+	+	–
3	pPTCL	–	+	+	+	–	+	–	–	+	+
4	pPTCL	–	+	+	+	–	–	–	+	+	–
5	pPTCL	–	+	+	+	–	+	–	+	+	+
6	pPTCL	–	+	+	+	–	–	–	+	+	+
7	UN	–	–	–	–	–	–	–	–	–	–
8	pPTCL	–	+	+	+	–	+	–	+	+	+
9	pPTCL	–	+	–	–	–	–	–	–	+	+

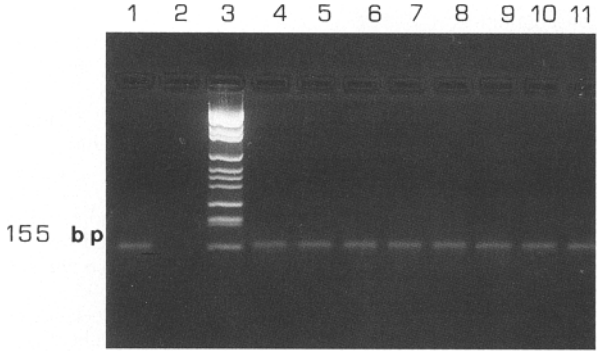

Fig. 1 Polymerase chain reaction amplification of Epstein-Barr virus (EBV) genome. Line 1 is the positive control; Line 2 is the negative control; Line 3 is the marker (Boehringer VI); Lines 4–11 are the EBV positive cases

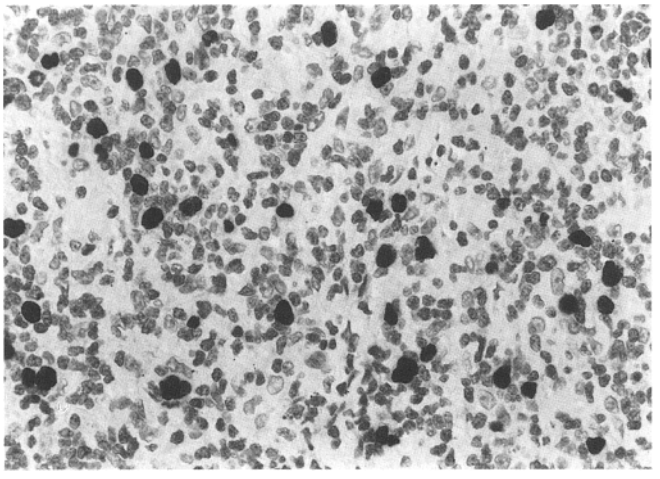

Fig. 2 Large atypical lymphoid cells are strongly labelled by EBV encoded RNAs-in situ hybridisation. ×350

observed in three cases. A cytoplasmic positivity was found with the same cellular distribution of EBER (Fig. 3). EBV was never detected by PCR or ISH in any of non-neoplastic control tissues nor in the epithelial cells present in the lymphomas.

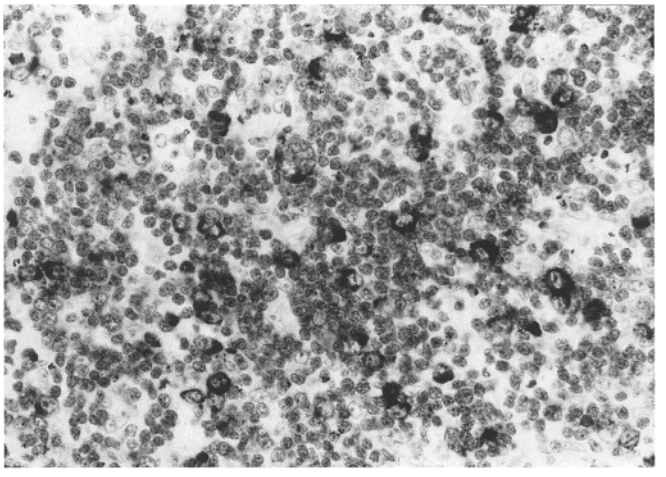

Fig. 3 Late membrane protein immunostaining was observed on the membrane and cytoplasm of large atypical lymphoid cell. Alkaline phosphatase anti-alkaline phosphatase ×375

Discussion

In a 10 year period (1983–1993) we have diagnosed eight cases of SNHLs. They represented 1.6% of all malignant lymphomas, 2.4% of extranodal malignant lymphomas and 10% of the extranodal malignant lymphomas of the head and neck. These percentages are in accordance with those reported by the majority of the authors [7, 9, 10, 32].

Eight of the nine cases showed a T-cell phenotype (one case gave inconclusive results). A clonal rearranged T-cell antigen receptor beta chain gene was observed by PCR in five cases. Molecular studies to assess the clonality of SNHL have been performed on only limited number of cases and the results have been contradictory [8, 13, 16, 17, 19, 30]. Different techniques have been used and the sensitivity of Southern-blot analysis, for example, ranges from 1 to 5%. PCR instead can detect monoclonality when the tumour DNA comprise only 0.05% of the sample [18]. Further studies are necessary to clarify these discrepancies.

The association of SNHL and EBV was accomplished in this study by the application of two different and independent nucleic acid detection methods: the PCR technique for the demonstration of EBV-specific DNA sequences and the ISH for the detection of EBER. The sensitivity of PCR was higher than that of EBER-ISH: eight positive cases with PCR, five positive cases with EBER-ISH. This may be due to single EBV infected cells expressing EBER at low concentration that might have escaped detection by ISH. EBV RNA was found mostly in the large atypical lymphoid cells, while the small cell component was usually negative. EBV probably plays a pathogenetic role if all the tumour cells are EBER positive. In those cases which show EBER positivity only in a portion of tumour cells, the virus might be an important but not essential co-factor in the multi-stage development of malignancy [33]. DNA analysis of single cells [3] may be useful in defining the question as to whether the large and small cell component belong to the same neoplastic clone and whether the demonstration of EBV infection in only a proportion of the tumour cells is due to down-regulation of EBER or elimination of the viral genome or a mixture of both.

LMP expression of EBV infected cells, although variable, is considered to be relevant because of its in vitro transforming potential as well as its capacity of prolonging cell survival by blockage of programmed cell death through induction of BCL2 expression [15]. However, BCL2 protein was never detected in our cases and LMP was found only in three cases.

In benign lymph nodes, the vast majority of EBV infected cells are B-lymphocytes; in contrast, there are now several reports of EBV infection of neoplastic cells in T-cell lymphomas, including sinonasal lymphomas. Asian countries and Peru have a markedly higher incidence of nasal lymphomas than the USA [21], and T-phenotype predominates in sinonasal lymphomas of these countries. A B-phenotype is more frequently encountered in sinonasal lymphomas in Western countries. In contrast our cases showed a T-cell phenotype and a TCRβR was also observed, at least, in some of them. EBV positivity has been consistently shown in T-cell sinonasal lymphomas, especially in Asian countries and Peru [2, 14, 16, 31]. However, minor series of EBV-positive B-cell sinonasal lymphomas have also been published; thus, it may be assumed that the anatomical site is important in the origin of EBV-associated lymphomas. The more constant presence of EBV infected cells in sinonasal lymphomas occurring in Asian countries (and in Peru) might be due to the earlier occurrence of EBV infection in these areas. In the control cases we examined, no EBV positivity was detected; thus, EBV infected cells in lymphomas of our series cannot be considered to be simply incidental. The results of our study suggest that EBV positive SNHL also occur in Italy and that EBV may have a role in the genesis of these malignancies, at least, in the sinonasal region.

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