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Short Communication

Epstein-Barr Virus Association in Pediatric Abdominal Non-Hodgkin-Lymphomas from Turkey

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Abstract. Recent sudies have shown marked geographic variation associated with Epstein-Barr virus (EBV) in pediatric Burkitt's lymphomas and Hodgkin's disease. In the present study we investigated 30 cases of pediatric extranodal high grade non-Hodgkin's lymphomas (NHL) from Turkey with an abdominal localisation. To classify them histologically and to determine the role of EBV in these lymphomas, immunohistochemistry (IHC), *in situ* hybridisation (ISH) and polymerase chain reaction (PCR) were used. Our series contained two histologic types: the Burkitt's or Burkitt's-like lymphomas (BL/BLL) and high grade NHL. They all were of the B cell type. The immunoglobulin heavy chain gene rearrangement revealed monoclonality in 87% of the BL/BLL cases, in contrast to the NHL cases, showing monoclonality in only 43% of the cases. EBV was found in tumor cells in a high frequency, independent of the histological subtype. EBV strains A and B were detected in 9 cases, with a preponderance of the B subtype (4/9 BL/BLL; 4/9 NHL). Our data suggest that high grade NHLs with abdominal localisation of Turkish children show the pattern of immunodeficient lymphomas to some extent.

Key words: Epstein-Barr virus; non-Hodgkin's lymphoma; pediatric; Turkey.

Introduction

Childhood non-Hodgkin's lymphoma (NHL) differs considerably from adult NHLs, both pathologically and clinically. Pediatric patients often present with high stage extranodal, disseminated and rapidly proliferating disease[16, 24, 25]. Abdominal localisation is very common. Histologically only three types of NHLs prevail, which contrasts with the adult situation[16, 24, 25]. The most frequent type is Burkitt's ltmphoma (BL) and/or Burkitt's – like lymphoma (BLL) (35–50%), followed by precursor lymphoblastic lymphomas/leukemias (30–40%) and diffuse large cell lymphomas (15–25%)[16]. The incidence of childhood NHL represents around 5% of all malignant neoplastic diseases of childhood in Western countries, whereas in equatorial Africa some subtypes, such as the endemic BL, account for nearly 50% of all childhood cancers in this area[1, 25]. Furthermore, NHLs other than endemic BL represent the most common childhood cancer in some countries, such as in Nigeria or Israel[1, 20]. There is an increasing incidence of NHLs in immunocompromised children due to congenital or acquired immunodeficient states[9, 7]. The peak incidence in immunocompetent children is between the ages of 7–11 years with a male preponderance (m : f = 4 : 1)[25]. BL occurs with a high incidence in equatorial Africa

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(the endemic form) and with a much lower frequency in Western countries (the sporadic form)[6]. Endemic BL is almost always associated with EBV and EBV DNA is present within the tumor cells of 95% or more of such tumors, compared with 10 to 20% in sporadic forms[14, 22]. A recent study demonstrated the presence of EBV genome in tumor tissues from Turkish patients in over 90% of cases, suggesting that BL in Turkish children appears to be similar to the African type[7]. Similar findings are known from Turkish cases of Hodgkin's disease (HD), where the occurrence of EBV expression is higher than in Western countries and similar to that quoted in the few available reports from other developing countries, such as China, Honduras and Peru with 61, 100 and 94%, respectively[2, 8, 26]. In contrast to the BL/BLL lymphomas, the remaining two histological types of NHL are not known to be related to infectious agents.

According to their DNA sequence (and protein) divergence within the EBV nuclear antigens (EBNA-2, -3a, -3b, -3c) and the EBV-encoded RNAs (EBER) region, two types of EBV (EBV type A, EBV type B) have been identified[21]. The biological differences between type A and type B appear to be mainly caused by the divergence within the EBNA-2 protein, because the type B strain transforms lymphocytes less efficiently than the type A strain and shows other differences in cell culture[19]. In general, EBV type A is the predominant type in lymphatic cells of EBV-infected cells. In human disease it now appears that the type B strain occurs preferentially in lymphoproliferative lesions that develop in HIV patients[5].

In the current study we report the clinical features, immunophenotype and EBV assessment of 30 cases of abdominal NHL in Turkish children.

Materials and Methods

Case selection. The tissues were retrieved from the files of the Children's Hospital, Hacepetteppe University, Ankara. They consisted of 30 cases of extranodal, abdominal NHLs, collected over a period of 17 years from 1978 to 1995. The samples included 27 male and 3 female patients, with a mean age of 4.8 years (1.5–11.0 years). As controls, reactive lymphoid lesions were investigated derived from gastrointestinal lymphoid tissue of 10 otherwise healthy individuals (6 male, 4 female patients, mean age of 5.0 years: 1 month–16 years). Clinical data were age, sex and primary tumor localisation (Table 1).

Histo-morphology. A first review of the cases was done on the basis of HE and Giemsa stained slides. Cases with valuable material were immunostained and diagnosed according to the REAL-classification[11]. All cases were further investigated as described in detail.

Table. 1. Clinical data

30 cases with NHL
27 male
3 female
mean age of 4.8 years (1.5–11 years)
10 cases with reactive lymphoid lesions in the GIT
6 male
4 female
mean age 5.0 years (1 month – 16 years)
Tumor localization
abdominal mass 7
small intestine 3
ileum 7
ileo-coecal mass 4
coecum 1
colon+ mesenterium 2
omentum/mesenterium 3
peritoneum 1
retroperitoneum 2

NHL – non-Hodgkin's-lymphoma, GIT – gastro intestinal tract.

Immunohistochemistry and in situ hybridisation. Immunohistochemistry was performed on formaldehyde-fixed paraffin-embedded tissue using the avidin-biotin complex method with diaminobenzidine as a chromogen. Primary antibodies were directed against terminal deoxynucleotidyltransferase (TdT), CD3, CD15, CD20, CD30, CD43, CD45, CD45R, CD45RO, CD79a, EMA and the latent membrane protein of EBV (LMP1). The proliferation rate was determined by using the antibody MIB1.

In situ hybridisation (ISH) for EBER1 (small nuclear non-transcribed mRNA: EBER1) was performed on formal – fixed paraffin embedded tissue sections as described previously[4]. The source of all the antibodies used was DAKO, Glostrup, Denmark.

PCR analysis for detection of EBV-genome and the immunglobulin heavy chain (IgH) gene-rearrangement. E B V-s t a t u s. General sequences of the EBV genome (gp220) as well as subtype specific sequences of the EBNA-2 and EBNA-3 regions were amplified after DNA extraction from whole tissue sections using a recently published protocol[4], slightly modified by use of a "hot start PCR" with Ampliwax™ PCR Gems (Perkin Elmer). The DNA quality was assessed using a β-globin amplification prior to EBV-specific PCR. Only samples with positive β-globin sequences were used for further PCR. The PCR comprised 37 cycles at 1 min 94˚C, 1 min 54˚C and 1 min 72˚C with three initial cycles at 1 min 97˚C, 3 min at 64˚C and 1 min at 72˚C; the initial de-

Table 2. DNA-sequences used for PCR

β-globin
PCO3: 5'ACACAACTGTGTTCACTAGC3'
PCO4: 5'CAACTTCATCCACGTTCACC3'
gp220 of EBV
gp220 1: 5'GGCTGGTGTCACCTGTGTTA3'
gp220 2: 5'CCTTAGGAGGAACAAGTCCC3'
Probe:
5'GGTGGAGGGGCTGAGTGTCTCTGGGTTTGAACTGG3'
EBNA-2
gen1: 5'AGGGATGCCTGGACACAAG3'
gen2: 5'GTGCTGGTGCTGCTGGTGG3'
EBNA-2 subtype 1
EBNA-2 A1: 5'TCTTGATAGGGATCCGCTAGGATA3'
EBNA-2 A2: 5'accgtggttctggactatctggat3'
Probe-5'CTCTGTCACAACCGAGGCTTACC3'
EBNA-2 subtype 2
EBNA-2 B1: 5'CATGGTAGCCTTAGGACATA3'
EBNA-2 B2: 5'AGACTTAGTTGATGCCCTAG3'
Probe-5'AGGCCTACTCTTCCTCAACCCAG3'
Immunglobulin heavy chain gene-rearrangement
IgH V: 5'CTGTCGACACGGCCGTGTATTACT3'
IgH J: 5'AACTGCAGAGGAGACGGTGACC3'

naturation step was 5 min (97˚C) and the terminal extension step was 10 min (72˚C). All amplifications were subjected to Southern blotting and hybridisation with digoxigenin-labeled – probes. DNA-extracts of the following cell lines were used as positive controls: Raji, AG876 (subtype B) and B95.8 (subtype A).

I g H - r e a r r a n g e m e n t. The DNA extracted as above was processed to PCR with the following conditions. The final reaction volume of 20 µl contained 50 mM KCl, 10 mM Tris-HCL (pH 8.3), MgCl 2.0 mM, 0.001% Gelatine, 0.1% TritonX-100, each dNTP at 200 µM, each primer at a final concentration of 2 µM and 0.5 unit of Taq DNA polymerase (Super Taq by Stehelin & Cie AG, Basel). Ampliwax™ PCR Gems separated the PCR-Mix into two phases: a lower phase, containing 10× buffer, dNTP's, primers and a small amount of water and an upper phase consisting of Taq DNA polymerase, the remaining water and the sample-DNA. Cycling conditions consisted of a touch down program, with an initial denaturation step of 2 min at 94˚C followed by 10 cycles with 30 s at 94˚C for melting, 30 s at 65˚C for annealing and 30 s at 72˚C for extension. At an annealingtemperature of 55˚C, 30 further cycles were carried out with 30 s at 94˚C and 20 s 55˚C at 72˚C and a final extension step was 7 min at 72˚C. Signals of 90–160 base pairs were analyzed on a polyacrilamide gel (8%). Only samples with positive β-globin sequences were used for further immunoglobulin gene-rearrangement – PCR.

The sequences of the primers used in this study are listed in detail in Table 2. We used primers as described recently[4, 23].

Results

In accordance with the REAL classification and the revised International Classification of Diseases (ICD-0-2), the series contained two histologic types which consisted of BL/BLL and high grade NHLs[11, 13]. The latter group was difficult to classify according to histological criteria and, therefore, these cases were listed together as NHL not otherwise specified (NOS). All the lymphomas investigated were of B cell origin ($CD20^+$, $CD79a^+$, $CD3^-$, IgH-rearrangement$^+$) and showed a high mitotic rate (40–80%). Nuclear staining for TdT, was negative in our cases. TdT is expressed in virtually all lymphoblastic lymphomas of B or Tcell origin, but not in other types of childhood NHL. The IgH gene-rearrangement revealed monoclonality in 87% of the BL/BLL cases in contrast to the NHL/NOS cases which were monoclonal in only 43% of the cases. In the latter group, the remaining cases were bi-, oligo- or polyclonal (Table 3).

The EBV association was investigated by different methods including immunohistochemistry for the latent membrane antigen, ISH for small nuclear non-transcribed mRNA and PCR for a general sequence of the glycoprotein gp220, and subtype A and B specific sequences. We found EBV-positive cases by ISH and/or PCR in 20 out of the 30 cases (67%) investigated. Taking into account the histologic subgroups, there was nearly equal EBV-positivity between the two groups: 14 out of the 20 BL/BLL (67%) and 7 out of 10 NHLs (70%) contained EBV. By means of ISH (EBER1) it was shown that EBV is diffusely expressed in over 90% of the tumor cell population (Fig. 1). Three out of 10 cases of NHL stained clearly for LMP. There was no LMP1 staining in the BL/BLL cases. In 9 cases we were able to determine EBV-subtypes. All these cases

Table 3. Immunoglobulin heavy chain gene-rearrangement

	BL/BLL	NHL/NOS
Monoclonal	13/15 (86.6%)	3/7 (42.9%)
Biclonal	1/15 (6.7 %)	1/7 (14.3%)
Oligoclonal	0/15 (0%)	1/7 (14.3%)
Polyclonal	1/15 (6.7%)	2/7 (28.5%)

BL – Burkitt's lymphoma, BLL – Burkitt's-like lymphoma, NHL – non-Hodgkin's lymphoma, NOS – not otherwise specified.

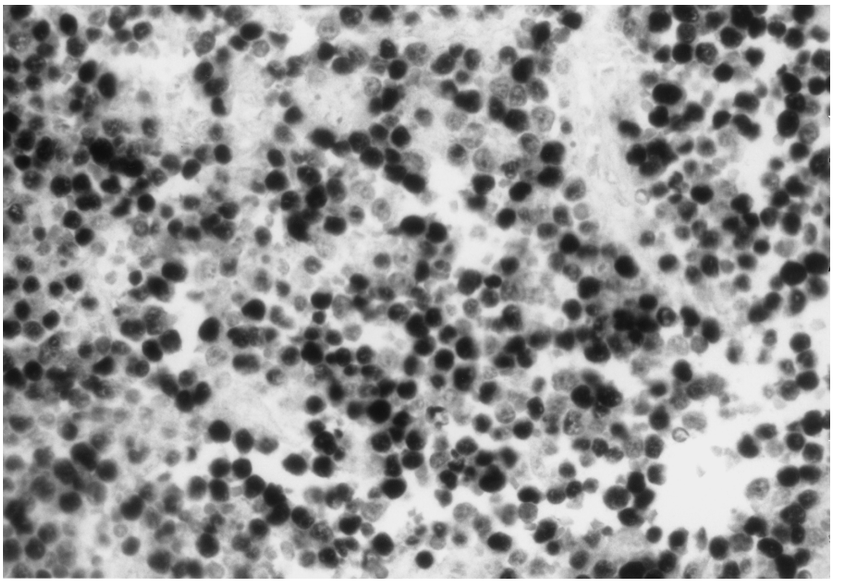

Fig. 1. *In situ* hybridisation of EBER showing a very high percentage of malignant cells expressing the early RNA of the Epstein-Barr virus

were positive for the house-houskeeping gene of β-globin. There was a preponderance in these cases of the B-subtype (4/9 BL/BLL; 4/9 NHL/NOS), with only one case of NHL/NOS (1/9) which was infected by the subtype A. There was no co-infection observed. EBV was not found by IHC, ISH or PCR in the ten control cases.

Discussion

We investigated 30 cases of pediatric NHLs from Turkish children. This cohort was particular for the fact that all presented with extranodal abdominal localisation at different sites (Table 3). Clinical data were available on age, sex and tumor localisation. Histologically, all cases were high grade B cell neoplasms and infiltrated the intestinal wall and surrounding structures often in a diffuse manner. They presented two histologic groups, with 20 of the 30 cases being BL or BLL (20/30) and 10 cases representing high grade NHLs/NOS (10/30). The abdominal localisation is not only the preferential site of Burkitt/Burkitt's-like lymphomas, but also, to a lesser extent, of high grade NHLs. In endemic, African, cases the typical localisation of BL is the jaw, an area near the entry of the EBV. In our series, the tumors were distributed along the whole small and large bowel. Both, jaw and bowel, are sites of intense contact of antigens with the immunesystem. In small children, the T and B cell immunesystems are immature[12]. Hence, we could speculate that a continuous antigen presentation in early childhood could lead to a perpetual proliferation of B cells and, thus, to lymphoma formation in this localisation. Almost all of our BL/BLL were monoclonal, whereas the NHL were more heterogenous in this respect, containing oligo-, bi- or monoclonal cases. EBV was associated in a high frequency as shown by ISH or PCR, independent on the histological subtype. In 9 cases we determined the EBV-subtypes. The B subtype was predominant and only one case was harboring the subtype A. The EBV subtype A has shown, when compared with subtype B, a higher transforming efficiency, a better initial outgrow and a lower cell-density dependence for cell viability *in vitro*[19]. In general the subtype A is the predominant type in lymphatic cells of EBV-infected individuals in the Western world. Subtype A has only rarely been detected in lymphoproliferative disorders, such as in angioimmunoblastic T cell lymphomas (AILD) of adults[4]. Immunocompromised patients, especially those with the acquired immunodeficiency syndrome, show an increased infection rate to the EBV-subtype B[3, 5]. A study with healthy Turkish children has shown an overall high seropositivety for EBV in this population[7]. The EBV-VCA seropositivity reached nearly 50% in children of 6 months and reached the highest level of 80% by 11 years of age. This could account for the high number of EBV in our tumors. However, different techniques employed, such as ISH (EBER), did not show EBV in the non-tumoral tissue of the control cases. EBV has been demonstrated in up to 100% of endemic BL and in only 15–30% of sporadic BL from the United States[22]. Therefore, the expression of EBV mRNA in nearly 90% of the tumor cells of our cases as well as the preponderance of the

EBV subtype B places these childhood NHL rather into the category of developing countries or of immunocompromised patients[5]. A similar finding could be observed in a recently published study by QUINTANILLA-MARTINÉZ et al.[18] which shows a higher frequency of EBV-positivitiy in primary intestinal lymphoma of Mexican origin as compared with Western European cases in a mixed population of adults and children.

The high EBV seropositivety in Turkish children occurring in early childhood could lead to an increased infection rate and proliferation of B cells. This renders the Bcells more susceptible to subsequent molecular events. So-called "second hits" might be necessary for a complete malignant transformation leading to different types of lymphoproliferation. BL are known to carry translocations or mutations of the *c-myc* gene, bcl-6 or p53. Our data suggest, even though we did not investigate the above mentioned genetic alterations, that similar mechanisms may be involved in high grade NHLs, other than BL in our series. It would be interesting to investigate *c-myc*, bcl-6 or p53 mutations in such cases; however, this was impossible in our study.

In conclusion, we investigated 30 high grade extranodal B cell NHL from Turkish children. Tissue data proved that EBV infection takes place early in childhood and contributes to the expansion of a B cell clone and malignant transformation early in childhood. All the cases showed, independently of the specific histologic subtype, an extranodal lymphoma manifestation, EBV association to a high frequency and, to a certain extent, a clonality pattern which brings them close to the pattern of immunodeficient lymphomas.

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