Expression of Epstein-Barr virus encoded latent genes in nasal T cell lymphomas

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Abstract

Aims—To determine the expression of Epstein-Barr (EB) virus encoded latent genes in nasal T-cell lymphomas in The Netherlands.

Methods—Seven euroid (Dutch) cases of nasal T cell lymphoma were investigated for the presence of EB virus by RNA in situ hybridisation (EBER). The expression of the EB virus encoded genes BARF0, EBNA1, EBNA2, LMP1, LMP2A, LMP2B, and ZEBRA was studied at the mRNA level using reverse transcriptase polymerase chain reaction. At the protein level the expression was investigated of EBNA2 and LMP1 by immunohistochemistry.

Results—In all seven nasal T cell lymphomas EBER was detected in the nuclei of virtually all tumour cells. BARF0 mRNA was detected in all samples. EBNA1 mRNA was found in six cases, LMP1 mRNA in five, LMP2A mRNA in three, LMP2B mRNA in one, and ZEBRA mRNA in one. EBNA2 mRNA was not found in any case. At the protein level occasional LMP1 positive tumour cells were seen in only one case. The EBNA2 protein was not detected.

Conclusions—Nasal T cell lymphomas in The Netherlands are strongly associated with EB virus. The virus shows a type II latency pattern (EBNA1+, LMP1+, EBNA2-) that seems to be similar to the EB virus associated nasal T cell lymphomas in oriental countries.

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type II is found, with expression of EBER 1/2, EBNA1, LMP1, BARF0, and often LMP2A or 2B.¹¹⁻¹⁶ Latency type III (EBER1/2, EBNA1, -2, -3A, -3B, -3C, -LP, LMP1, -2A, -2B, and BARF0) is present in large B cell lymphomas in immunocompromised individuals.^{17,18}

There are two different promoter sites for the transcription of EBNA1, the C/W promoter and the F promoter. The F promoter is active in Burkitt's lymphomas and in nasopharyngeal carcinomas (latency type I and II) and results in the Q/U/K splice variant of EBNA1 mRNA, while the C/W promoter is active in type III latency, resulting in the Y3/U/K splice variant.^{19,20}

Studies on the expression of EB virus encoded gene products in nasal T cell lymphomas have provided conflicting results, both at the protein level and at the mRNA level. Not all studies agree on the expression of the EB virus encoded protein latent membrane protein 1 (LMP1) and EB virus nuclear antigen 2 (EBNA2) in these lymphomas. Expression of these proteins may be important, since they are known for their transforming capacities in vitro.^{21,22}

This prompted us to investigate the expression of EB virus latent genes in cases of nasal T cell lymphoma from The Netherlands. We also investigated the transcription of BZLF1 transactivation protein (ZEBRA). ZEBRA mediates a switch between the latent and lytic state of EB virus infection by binding, as a dimer, to the promoters of genes involved in lytic DNA replication and activating their transcription.^{23,24}

Methods

Formalin fixed, paraffin wax embedded material and snap frozen tissue stored at -80°C of seven cases of nasal T cell lymphoma were retrieved from the files of the pathology departments of the University Hospital of Utrecht and the Free University Hospital of Amsterdam. Clinicopathological and immunophenotypic findings of these cases have been published recently.⁸

IN SITU HYBRIDISATION

The presence of EB virus was studied in paraffin embedded tissue sections using a very sensitive RNA in situ hybridisation against EBER 1 and 2, two EB virus encoded RNAs that are expressed in high quantity in latently infected cells. In three cases a fluorescein conjugated

Recent reports have shown a strong association between nasal T cell lymphomas and Epstein-Barr (EB) virus in both oriental and occidental countries, suggesting that the virus plays a role in the aetiology of this neoplasm.¹⁻⁸ EB virus is known to be associated with several different malignancies. The pathogenic role of this virus in these neoplasms, if any, is not fully understood, but it is probably different in the various tumours, since the expression of EB virus encoded latent gene products is not identical.

Three different patterns of EB virus latent gene expression are known to be associated with the various EB virus associated malignancies. Type I latency, that is, expression of EBER 1/2, EBNA1, and BARF0, is found in African Burkitt's lymphoma.^{9,10} In Hodgkin's disease and nasopharyngeal carcinoma, latency pattern

Table 1 Sequences of PCR primers and oligoprobes

Transcript	Oligo ¹	Sequence (5'-3')	Amplimer length
BARF0	A3 (s)	AGAGACCAGGCTGCTAAACA	240 bp
	A4 (as)	AACCAGCTTTCCCTTTCCGAG	
EBNA1	Probe	AAGACGTTGGAGGCACGCTG	Y3/U/K spliced: 265 bp Q/U/K spliced: 236 bp
	Y3 (s)	TGGCGTGTGACGTGGTGTA	
	Q (s)	GTGCGCTACCGGATGGCG	
	K (as)	CATTTCCAGGTCTGTACCT	
EBNA2	U (probe)	AGAGAGTAGTCTCAGGCGAT	195 bp
	Y2 (s)	TACGCATTAGAGACCACTTTGAGCC	
	H1 (as)	AAGCGCGGGTGCTTAGAAGG	
	Y3 (probe) ²	TGGCGTGTGACGTGGTGAA	
LMP1	1 (s)	TGTACATCGTTATGAGTGAC	240 bp
	2 (as)	ATACCTAAGA/CAAGTAAGCA	
	3 (probe)	ACAATGCCTGTCCGTGCAAA	
LMP2A	A1 (s)	ATGACTCATCTCAACACATA	280 bp
	AB2 (as)	CATGTTAGGCAAATTGCAAA	
	Probe	ATCCAGTATGCCTGCCTGTA	
LMP2B	B1 (s)	CAGTGTAACTGTCACAAAGA	324 bp
	AB2 (as) ³	CATGTTAGGCAAATTGCAAA	
	Probe	ATCCAGTATGCCTGCCTGTA	
ZEBRA	Z1 (s)	CGCACACGGAACACAAACAGC	227 bp
	Z2 (as)	CGGCGGATAATGGAGTCAACATCC	
	Probe	GCTTGGGCACATCTGCTTCAACAGG	

¹ The sense (s) or antisense (as) orientation is indicated in brackets.
² EBNA2 specific transcripts were detected with the Y3 probe, which was also used as primer for detection of EBNA1 transcripts.
³ The antisense primer and probe of LMP2B are similar to those of LMP2A.

mixture of oligoprobes was used against EBER 1 and 2 (Dako, Glostrup, Denmark), and in four cases a mixture of EBER specific biotinylated EBER 1 and 2 RNA antisense or sense (control) (kindly provided by Dr L S Young, Birmingham, United Kingdom). The in situ hybridisation protocols have been described recently.^{25,26} An EB virus positive Hodgkin's disease specimen served as a positive control.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

The reverse transcriptase polymerase chain reaction (RT-PCR) protocol has been published recently.²⁷ In short, ten 10 µm sections were cut from snap frozen tissue samples and homogenised in an RNazol B buffer. RT-PCR was performed using intron flanking primers. The primers sequences and oligoprobes used for analysis have been published previously and are listed in table 1.^{11,13,27,28} For the RT-PCR an amount of RNA was used equivalent to RNA extracted from one 5 µm section. RT reaction was performed in a final volume of 20 µl containing 25 pmol of one to four of each EB virus antisense primer specific for the different genes. To exclude false positive signals resulting from DNA amplification, simultaneous reactions were performed with omission of the reverse transcriptase. After denaturation of the DNA, 40 cycles of amplification were performed. The amplification products were analysed by electrophoresis on agarose gel and hybridised overnight with a specific ³²P end labelled internal oligoprobe. The EB virus transformed lymphoblastoid KCA cell line and the EB virus negative B cell line BJAB were used as positive and negative controls.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on 6 µm sections of the snap frozen material. The

primary antibodies used were raised against EBNA2 (PE2, Dako) and LMP1 (CS1-4, Dako). Either an avidin-biotin HRP complex method or an indirect alkaline phosphatase technique was used. A lymph node from a patient with infectious mononucleosis served as a control.

Results

The nuclei of virtually all the tumour cells in all seven cases stained positive after the EBER in situ hybridisation. Epithelial cells, endothelium, and reactive non-malignant infiltrate were negative (fig 1).

The sensitivity of the RT-PCR protocol for EBNA1, EBNA2, and LMP1 was estimated by using a dilution series of the EB virus transformed lymphoblastoid KCA cell line in EB virus negative BJAB B lymphocytes (A Brink *et al*, manuscript submitted). Detection of up to 5 KCA cells was possible in a background

Figure 1 Positive signal in the nuclei of the atypical cells after EBER in situ hybridisation. Note that the nuclei of the epithelial cells and the reactive infiltrate of small mononuclear cells are negative.

Figure 2 Blot of LMP1 RT-PCR products of a dilution series of KCA cells (EB virus positive) in BJAB cells (EB virus negative).

of 5×10^5 BJAB cells after hybridisation and 12 hours of autoradiography (fig 2). The results of RT-PCR are shown in table 2. Although the function of the BARF0 transcript is not known, it seems to be transcribed in high quantities in all cells latently infected with EB virus.¹⁰ BARF0 was detected in all specimens, thereby confirming the in situ hybridisation results which also showed that EB virus was present in all specimens and that mRNA was available for RT-PCR (fig 3). In all samples but one EBNA1 mRNA was found. The signal was weak in two samples (fig 3). In these six cases the Q/U/K splice variant was present. The Y3/U/K splice variant was not found in any case. In none of the seven cases was EBNA2 detected, but in five cases LMP1 mRNA was present (fig 3). LMP2A was found in three cases and LMP2B in one. A faint positive signal of ZEBRA mRNA could be detected in one lymphoma.

As with the RT-PCR results, EBNA2 could not be detected at the protein level in any case. In only one of the five LMP1 mRNA positive cases (case 1) was this protein detected on occasional tumour cells. In the LMP1 mRNA negative cases the LMP1 protein was not detected.

Discussion

Although several recent publications describe a strong association between nasal T cell lymphomas and EB virus, little is known about the pathogenic role of the virus in this lymphoma, and there are conflicting reports on the expression of the EB virus encoded latent genes. Expression of these genes may be important. EBNA2 and LMP1 in particular are known for their transforming capacities in vitro and are thought to play an oncogenic

Figure 3 Blots of RT-PCR products LMP1, EBNA1, and BARF0 from the seven nasal T cell lymphoma samples. In cases 1 and 2 only a very faint EBNA1 signal was found.

role in the development of the various malignancies.^{21,22} The LMP2 proteins probably play a role in transmembrane signal induction.²⁹

In one article about a small series of nasal T cell lymphomas expression of both LMP1 and EBNA2 is reported at the protein level in all investigated cases,¹ whereas in another study only the presence of LMP1 could be detected, without expression of EBNA2.⁴ Others did not detect any LMP1 expression,³ or found positivity only in a minority of cases.⁵

Similarly, at the mRNA level (using RT-PCR) conflicting reports have been published. Minarovits *et al* found RNA coding for EBNA1 and LMP1 but not EBNA2 in all six nasal T cell lymphomas tested.³⁰ In contrast Suzushima *et al* only detected EBNA1 and not EBNA2 or LMP1.³¹ Interestingly, both studies comprised only Japanese cases. To the best of our knowledge there are no reports on EB virus gene expression in nasal T cell lymphomas at the mRNA level in europid cases.

Table 2 EBV gene transcription as determined by RT-PCR in diffuse EBER1/2 positive nasal T cell lymphomas

Case No.	EBNA1	EBNA2	LMP1	LMP2A	LMP2B	ZEBRA	BARF0
1	Q/U/K	—	¹	+	—	—	+
2	Q/U/K	—	+	+	—	—	+
3	—	—	—	—	—	—	+
4	Q/U/K	—	+	—	+	—	+
5	Q/U/K	—	+	—	—	—	+
6	Q/U/K	—	—	+	—	+	+
7	Q/U/K	—	+	—	—	—	+

¹ In this case the expression of LMP1 was confirmed by immunohistochemical detection of LMP1.

The present study supports a type II latency pattern in nasal T cell lymphomas in The Netherlands. Only the F promoter was used for the transcription of EBNA1 (Q/U/K splice variant); in most cases LMP1 mRNA was detected, while EBNA2 was constantly negative. EBNA1 mRNA was detected in almost all lymphomas. However, in one of our cases EBNA1 mRNA could not be detected. The most plausible explanation for this is that the relatively low expression level of EBNA1 mRNA was below the detection level of our RT-PCR technique in this case, possibly because of some degradation of mRNA in the tissue sample.

The pattern of EB virus gene expression in nasal T cell lymphomas in The Netherlands is probably similar to Japanese cases. In two of our seven cases, LMP1 mRNA could not be detected, and this might explain the results of Suzushima's small series,³¹ in which all three cases were negative for LMP1 mRNA. In most of our cases the pattern was similar to the cases described by Minarovits.³⁰

An EB virus latency type II expression is also found in Hodgkin disease and in undifferentiated nasopharyngeal carcinoma. However, there are some differences between Hodgkin's disease and nasal T cell lymphomas. In EB virus associated Hodgkin's disease, LMP1 mRNA can be detected in all EB virus positive samples, and more importantly, all malignant cells (Reed-Sternberg cells and variants) show strong expression of LMP1 at the protein level.^{11,12} In our study of nasal T cell lymphomas, on the other hand, LMP1 mRNA could not be detected in all cases. We cannot exclude the possibility that the LMP1 mRNA was degraded in some cases and therefore could not be detected. Still, in only one of the five LMP1 mRNA positive cases were occasional LMP1 positive tumour cells detected at the protein level. Recently, we have reported similar results on LMP1 expression at the protein level in a series of Chinese cases of nasal T cell lymphoma.⁵ The expression of EB virus encoded gene products in nasal T cell lymphomas shows more similarities to EB virus associated nasopharyngeal carcinoma, in which LMP1 mRNA expression is not found in all cases.¹³ Moreover, only in part of the LMP1 mRNA positive cases can the protein can be detected in occasional tumour cells by immunohistochemistry.¹³⁻¹⁶ Whether in the other LMP1 mRNA positive cases there is no translation to the protein level or whether the expression is below the level of detection has not been elucidated so far.

In Hodgkin's disease occasional Reed-Sternberg cells have been shown to express ZEBRA at the protein level, and similar findings have been described for nasopharyngeal carcinomas.^{32,33} The ZEBRA mRNA detected in case 6 suggests that occasionally the ZEBRA protein may be expressed in nasal T cell lymphomas as well. One can speculate that in occasional tumour cells a switch is made from latent to lytic cycle, and that occasionally viral particles will be produced. However, expression of the ZEBRA protein does not automatically

mean that a complete lytic cycle is taking place.³⁴ In the ZEBRA positive cases of Hodgkin's disease no expression of other lytic proteins was found (that is, EB virus early antigen, EA; viral capsid antigen, VCA; and membrane antigen, MA), suggesting an abortive viral lytic cycle.³² Although the expression of other lytic EB virus proteins in nasopharyngeal carcinomas is still under investigation, preliminary results also suggest an abortive lytic cycle.³³ Therefore it may well be that the presence of ZEBRA mRNA in one of the cases of nasal T cell lymphomas in our series is an expression of an abortive lytic cycle, and it does not necessarily mean that viral particles are produced.

In conclusion, nasal T cell lymphomas are strongly associated with EB virus. The virus shows a type II latency pattern, which seems to be similar to the EB virus gene expression in undifferentiated nasopharyngeal carcinomas. The EB virus gene expression pattern in nasal T cell lymphomas from The Netherlands does not differ from oriental cases.

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