Expression of bcl-2 and p53 proteins in nasopharyngeal carcinoma. Absence of correlation with the presence of EBV encoded EBER1-2 transcripts and latent membrane protein-1

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Abstract

***Aims*—To investigate the immunohistochemical expression of bcl-2 and p53 proteins in nasopharyngeal carcinomas in relation to the expression of the Epstein–Barr virus (EBV) encoded EBER messenger RNAs (mRNAs) and latent membrane protein-1 (LMP-1).**

***Methods*—Formalin fixed, paraffin wax embedded tissue from 44 nasopharyngeal carcinomas (NPCs) was stained by immunohistochemistry for p53, bcl-2 and LMP-1 proteins and by RNA in situ hybridisation for EBER mRNAs.**

***Results*—The tumours were divided histologically into 13 cases of keratinising squamous cell NPC (KNPC), 15 cases of non-keratinising squamous cell NPC (NKNPC) and 16 cases of undifferentiated NPC (UNPC). Bcl-2 expression was observed in five of 15 NKNPC cases and in six of 16 UNPC cases; p53 expression was observed in one of 13 KNPC, two of 15 NKNPC and four of 16 UNPC cases. EBER 1-2 transcripts were detected in five of 15 NKNPC and nine of 16 UNPC cases, while LMP-1 expression was observed in one of 16 UNPC cases. All 13 KNPCs were EBV and bcl-2 negative. No correlation was found between the presence of EBER 1-2 transcripts and the detection of bcl-2 or p53 proteins, or both, in NPC cells.**

***Conclusions*—The expression of bcl-2 and p53 proteins may be associated with the level of the tumour cell differentiation in NPC. In addition, in view of the important role of the bcl-2 protein in the inhibition of apoptosis, the expression of bcl-2 protein may contribute to tumour cell survival in a proportion of NPCs. Furthermore, in the light of previous findings that the p53 gene in most UNPCs is in the wild-type configuration, mechanisms other than mutation may be responsible for stabilisation of the p53 protein in UNPCs.**

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Keywords: Epstein–Barr virus, bcl-2, p53, nasopharyngeal cancer.

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The bcl-2 oncogene has been detected as a transcriptionally active unit on chromosome 18 in the vicinity of the breakpoint of translocation t(14;18) carrying follicular lymphomas.[1–3] The translocation leads to unregulated elevated expression of an otherwise unchanged bcl-2 protein.[4] This protein functions in an antioxidant pathway to prevent programmed cell death (apoptosis) and is localised to intracellular sites of oxygen free radical generation, including mitochondria, endoplasmic reticula and the nuclear membrane.[5] Using immunohistochemistry, bcl-2 protein has been detected in B and T non-Hodgkin's lymphomas lacking the t(14;18) translocation.[6] In addition, bcl-2 protein has been detected in epithelial malignancies and is reported to have prognostic value.[7–9]

P53 is thought to act as a tumour suppressor gene and is located on the short arm of chromosome 17. It encodes a 53 kilodalton phosphoprotein which is involved in the negative regulation of cellular growth by controlling entry of the cell into S phase.[10 11] Although normal p53 protein functions as a regulator of cell proliferation and inhibits transformation, point mutations can transform it into a dominant oncogene with transforming activity.[12] Mutations or deletions of the p53 gene have been detected in several types of human malignancies.[13–22] In normal cells the p53 protein has a very short half-life. By contrast, p53 gene mutations generally lead to stabilisation of the protein, which can then be detected using immunohistochemistry. Stabilisation may, however, be achieved through binding to other cellular or viral proteins.[23–25] Of particular interest is the relation between p53 and bcl-2 proteins. While bcl-2 is believed to be important in suppressing apoptosis,[6] wild-type p53 is thought to induce apoptosis under certain conditions.[12] Inactivation of the p53 gene could result in an oncogenic effect similar to that observed when bcl-2 protein, which suppresses apoptosis, is overexpressed.[6 12] In this regard, a recent immunohistochemical study on non-Hodgkin's lymphomas showed a significant inverse correlation between p53 and bcl-2 protein expression.[26]

Epstein–Barr virus (EBV) is a human herpes virus which immortalises normal B cells as permanent B lymphoblastoid cell lines.[27] EBV is believed to be involved in the pathogenesis of endemic Burkitt's lymphoma, nasopharyngeal carcinoma (NPC) and lymphoproliferative dis-

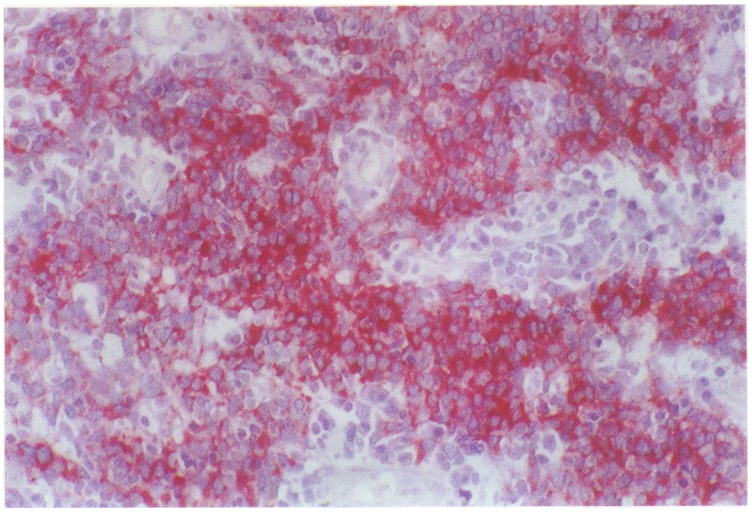

Figure 1 Bcl-2 protein staining in undifferentiated NPC tumour cells.

orders in immunosuppressed patients.[28-36] Recently, special attention has been paid to the EBV encoded latent membrane protein-1 (LMP-1) because of its transforming activity in vitro. Indeed, LMP-1 is capable of transforming fibroblastoid cell lines and rendering them tumorigenic in nude mice.[37] LMP-1 also inhibits differentiation of epithelial cells in vitro and induces morphological transformation of these cells.[38 39] Thus, LMP-1 may play a role in the development of the malignant phenotype of NPC.[40]

The relation between EBV and oncogenes is noteworthy. LMP-1 induces the expression of the bcl-2 protein in vitro[41] and p53 protein can bind the EBNA-5 protein.[42] The above observations prompted us to investigate the expression of bcl-2 and p53 proteins in the different histological types of NPC. We also attempted to define any association between the expression of the bcl-2 and/or p53 proteins and the presence of EBV in NPC. To this end we used immunohistochemical and RNA in situ hybridisation (RISH) techniques on formalin fixed, paraffin wax embedded tissue.

Methods

A search for NCPs diagnosed between 1978 and 1988 in the files of the Pathology Department, Evagelismos Hospital, Athens, and between 1990 and 1993 in the files of the Departhment of Pathology, University Hospital, Heraklion, yielded 44 cases. The patient population comprised 31 men and 13 women aged between 20 and 80 years. Diagnosis and histological classification were performed on formalin fixed, paraffin wax embedded sections, stained with haematoxylin and eosin, according to the World Health Organisation (WHO) classification.[43]

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using the alkaline phosphatase–antialkaline phosphatase (APAAP) method, as described previously,[44] for the presence of bcl-2, p53 and LMP-1 proteins. Mouse monoclonal anti-LMP-1 antibodies (CS1–4), bridging rabbit antimouse (Z 259) and APAAP complexes (D 314) were obtained from Dako. The anti-LMP-1 antibody was used at a dilution of 1 in 200 in TRIS (pH 7·6). The anti-bcl-2 (Dako) and the anti-p53 (1801) (Oncogene Sciences) monoclonal antibodies were used at dilutions of 1 in 30 and 1 in 100, respectively. The second and the third steps of the immunohistochemical procedure were repeated in all experiments to enhance the signal. Positive control slides were included in all tests and consisted of paraffin sections from Hodgkin's disease known to be positive for LMP-1, bcl-2 or p53 proteins. Negative control slides were prepared by omitting the primary antibody. In all cases the quality of antigenic preservation was tested by staining for vimentin.

RNA IN SITU HYBRIDISATION

This procedure was performed for the presence of EBER 1 and 2 messenger RNAs (mRNAs) using fluorescein isothiocyanate (FITC) oligonucleotides obtained from Dako (Y 017), as described previously.[45] Briefly, deparaffinised sections were rehydrated and pretreated with proteinase K, dehydrated, and air-dried. These were then hybridised for two hours at 37°C with the FITC conjugated EBER oligonucleotides in hybridisation solution consisting of 30% formamide, 10% dextran sulphate, 0·1% sodium pyrophosphate, 0·2% polyvinyl pyrrolidone, 0·2% ficoll, 5 mmol/l Na_2EDTA, and 50 mmol/l Tris/HCl (pH 7·5). After washing in Tris buffered saline (TBS), pH 7·6, containing 0·1% Triton X-100, the following immunohistochemical detection system was used: mouse anti-FITC, rabbit antimouse immunoglobulin, and APAAP complexes (Dako SA). Visualisation of the reaction was performed using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT). As a positive control a case of Hodgkin's disease known to be EBER positive was used. As negative controls, the hybridising mixture was used without EBV probes. In all cases the quality of RNA preservation was tested using poly dT oligonucleotides.

Results

HISTOLOGY

Nasopharyngeal carcinomas were classified as keratinising squamous cell carcinoma (WHO 1; 13 cases), non-keratinising squamous cell carcinoma (WHO 2; 15 cases) and undifferentiated carcinomas (WHO 3; 16 cases).

Table 1 Results of immunohistochemistry and RISH

Classification	*Number of cases*	*LMP-1 positive*	*bcl-2 positive*	*p53 positive*	*EBER 1–2 positive*
WHO 1	13	0	0	1	0
WHO 2	15	0	5	2	5
WHO 3	16	1	6	4	9

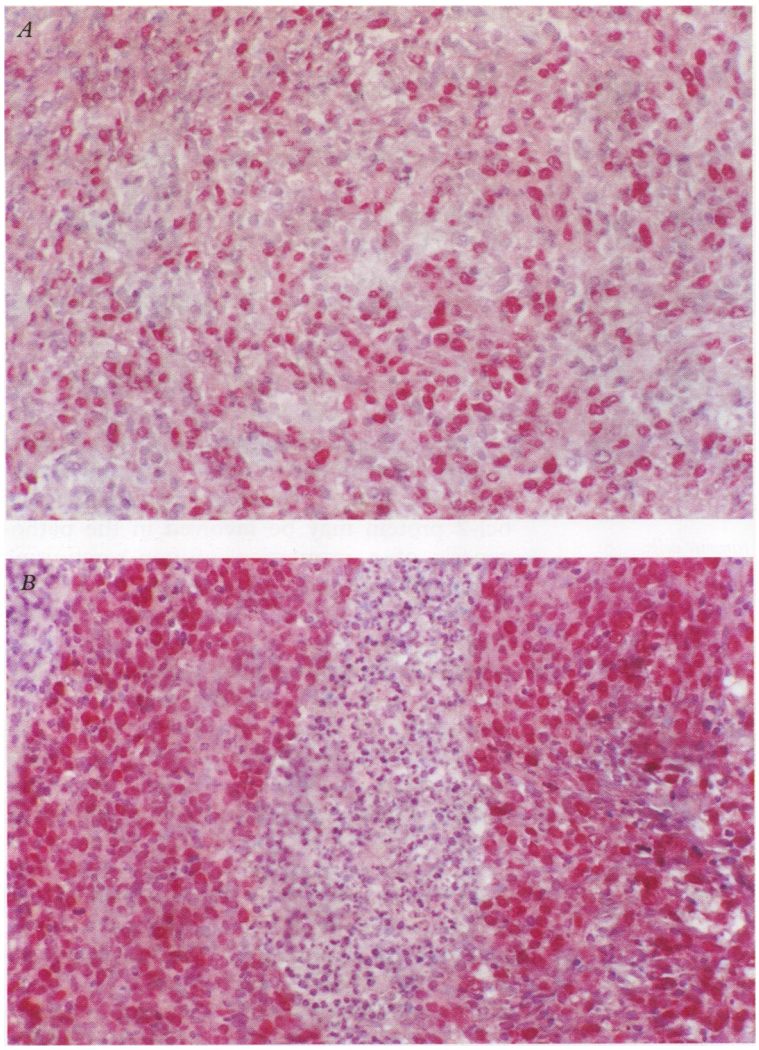

Figure 2 A: p53 protein staining in undifferentiated NPC tumour cells. B: p53 protein staining in tumour cells of squamous cell non-keratinising NPC.

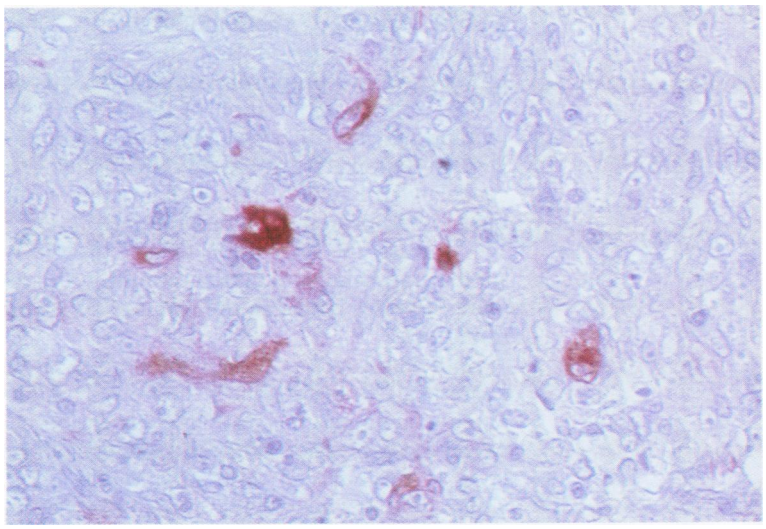

Figure 3 LMP-1 protein staining in undifferentiated NPC tumour cells.

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Bcl-2 positive staining was observed in 11 of 44 (25%) NPC cases (fig 1). In correlating bcl-2 expression with histological type positive staining was observed in five of 15 non-keratinising squamous cell carcinomas and in six of 16 undifferentiated carcinomas, the 13 cases of keratinising squamous cell carcinoma being bcl-2 negative (table 1). The type of staining in the neoplastic cells was cytoplasmic and the intensity of staining in these cells was stronger than that in the infiltrating small lymphocytes. Positive staining was observed in most normal basal epithelial cells present in the tumour biopsy specimens.

P53 nuclear staining was found in seven of 44 (15·9%) NPC cases (fig 2). In correlating p53 expression with histological type positive staining was observed in one of 13 keratinising squamous cell carcinomas, two of 15 non-keratinising squamous cell carcinomas and in four of 16 undifferentiated carcinomas (table 1). Nuclear staining was more distinct at the periphery of the tumour cells giving a palisading configuration. No immunoreactivity was found in normal nasopharyngeal epithelia, stromal cells or lymphoid cells.

Immunohistochemical analysis for EBV encoded LMP-1 expression in 44 NPC cases revealed unequivocal positive membrane and cytoplasmic staining in only one case of undifferentiated carcinoma (table 1) (fig 3). The normal epithelium present in the slide was LMP-1 negative.

RNA IN SITU HYBRIDISATION

The RISH technique demonstrated EBV EBER-1 and EBER-2 mRNAs in 14 of 44 (31·8%) NPC cases (fig 4). This technique also revealed EBV EBER 1–2 mRNAs in five of 15 non-keratinising squamous cell carcinomas and in nine of 16 undifferentiated carcinomas. All 13 cases of keratinising squamous cell carcinoma were negative (table 1).

RELATION BETWEEN BCL-2 AND P53 EXPRESSION

P53 positive cases accounted for three of the 33 bcl-2 negative and four of 11 bcl-2 positive cases. The co-expression of bcl-2 and p53 was statistically significant with a χ^2 value of 4·58 ($p<0·05$) (table 2).

RELATION BETWEEN EBER 1–2 AND BCL-2 EXPRESSION

Bcl-2 positive cases accounted for six of 14 EBER 1–2 positive cases and five of 30 EBER 1–2 negative cases. The difference was not statistically significant (table 3).

RELATION BETWEEN EBER 1–2 AND P53 EXPRESSION

P53 positive cases accounted for three of 14 EBER 1–2 positive cases and four of 30 EBER 1–2 negative cases. The difference was not statistically significant (table 4).

Discussion

In the present study bcl-2 protein expression was detected in six of 16 (37%) cases of undifferentiated and in five of 15 (33%) cases of

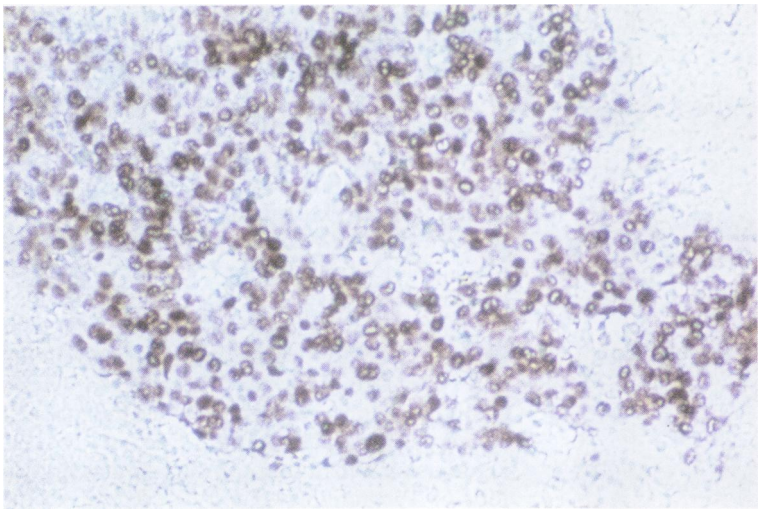

Figure 4 EBER 1–2 transcripts present in undifferentiated NPC tumour cells.

Table 2 Comparison of p53 and bcl-2 expression

Classification		*p53 positive*	*p53 negative*	*Total*
WHO 1	bcl-2 positive	0	0	0
	bcl-2 negative	1	12	13
WHO 2	bcl-2 positive	1	4	5
	bcl-2 negative	1	9	10
WHO 3	bcl-2 positive	3	3	6
	bcl-2 negative	1	9	10

Table 3 Correlation between EBER 1–2 and bcl-2 expression

Classification		*bcl-2 positive*	*bcl-2 negative*	*Total*
WHO 1	EBER 1–2 positive	0	0	0
	EBER 1–2 negative	0	13	13
WHO 2	EBER 1–2 positive	2	3	5
	EBER 1–2 negative	3	7	10
WHO 3	EBER 1–2 positive	4	5	9
	EBER 1–2 negative	2	5	7

Table 4 Correlation between EBER 1–2 and p53 expression

Classification		*p53 positive*	*p53 negative*	*Total*
WHO 1	EBER 1–2 positive	0	0	0
	EBER 1–2 negative	1	12	13
WHO 2	EBER 1–2 positive	1	4	5
	EBER 1–2 negative	1	9	10
WHO 3	EBER 1–2 positive	2	7	9
	EBER 1–2 negative	2	5	7

non-keratinising squamous cell NPC, whereas bcl-2 protein was undetectable in these cells. These results are in keeping with those reported recently by Lu *et al.*[46] Previous studies have shown that the bcl-2 gene is consistently rearranged in the t(14;18) translocation present in most follicular lymphomas and in a small proportion of diffuse large cell lymphomas.[1–5] Interestingly, the t(14;18) translocation can also occur with low frequency in benign follicular B cell hyperplasia.[47] Bcl-2 transgenic mice develop follicular hyperplasia, which may progress to high grade monoclonal B cell lymphoma in some instances.[48] This suggests that bcl-2 gene deregulation may be an important but not the sole factor involved in the development of B cell lymphoma.[48] As about 50% of B cell lymphomas in bcl-2 transgenic mice have c-*myc* gene rearrangements, it has been suggested that bcl-2 expression, by prolonging B cell survival, provides an opportunity for other genetic alterations to occur resulting in the development of overt lymphoid malignancies.[48] Thus, it could be hypothesised that bcl-2 protein may be involved in the pathogenesis of a proportion of non-keratinising squamous cell and undifferentiated NPCs by giving a survival signal to an epithelial cell clone (that is, from bcl-2 positive basal cells of the normal nasopharyngeal epithelium) and enabling it to persist until other signals (that is, activation of other oncogenes and/or inactivation of onco-suppressor genes) propagate the clone to malignancy. In this regard the relation between bcl-2 and p53 could be of interest. While wild-type p53 can induce apoptosis, inactivation of p53 by mutation could result in an oncogenic effect similar to that observed when bcl-2 is overexpressed—that is, inhibition of apoptosis.[6 12 26] In our study we observed p53 protein expression in four of 16 (30%) undifferentiated NPC cases, in two of 15 (13%) non-keratinising squamous cell NPC cases, and in one of 13 (7%) keratinising squamous cell NPC cases. These findings, in keeping with those reported by Niedobitek *et al*,[49] are of interest in view of previous data that the p53 gene in undifferentiated NPC is consistently in germ line configuration, whereas many squamous cell carcinomas of the head and neck display p53 gene mutations.[50–54] Thus, it could be suggested that detection of the p53 protein in a proportion of undifferentiated NPCs reflects stabilisation of the p53 protein by binding with other proteins rather than p53 gene mutation. In this regard, Niedobitek *et al* speculated that EBV encoded proteins could bind to p53 protein in undifferentiated NPC.[49] By contrast, the expression of p53 protein in some keratinising and non-keratinising squamous cell NPCs could be the result of p53 gene mutations.[50–54]

Both bcl-2 and p53 proteins tended to be expressed in histologically less well differentiated subtypes, suggesting a correlation between the expression of these proteins and the level of tumour cell differentiation in NPC. This could be related to recent observations that bcl-2 protein is expressed in a cell differentiation dependent manner in normal nasopharyngeal epithelia.[46]

In the present study we have confirmed previous findings that undifferentiated NPC is much more frequently associated with EBV than squamous cell NPC.[40 46 49] However, it is worth noting that we detected EBER 1–2

transcripts in a proportion of non-keratinising squamous cell NPCs whereas Niedobitek *et al*[35] did not. This difference may reflect epidemiological differences and/or slightly different criteria for the histological classification of NPC. Furthermore, we found EBER transcripts in nine of 16 undifferentiated NPC cases whereas previous studies detected EBV in most of these cancers.[40 46 49] This difference could be because of poor preservation of RNA in some of our cases. We observed LMP-1 expression only in one of 16 (6%) cases of undifferentiated NPC. Previous studies reported LMP-1 positivity in tumour cells in 20 to 30% of undifferentiated NPC when frozen tissue was used,[40 49] whereas in one study, which used fixed tissue, no LMP-1 expression was observed in 15 cases of undifferentiated NPC.[46] It is possible that these differences reflect case selection and/or problems caused by tissue overfixation, although the epitope recognised by CS 1–4 monoclonal antibodies is considered to be fixation resistant.

In the present study we found no correlation between the presence of EBV EBER mRNAs and bcl-2 or p53 protein expression in NPC. This is in keeping with the previous findings reported for Hodgkin's disease and cutaneous T cell lymphomas.[55-59] However, these findings do not exclude the possibility that EBV encoded proteins, other than LMP-1, influence the expression of the bcl-2 and p53 proteins in NPC. Further studies are needed to unravel any correlation between EBV and the expression of oncogenes and onco-suppressor genes in NPC.

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