Loss of p16 Expression Has Prognostic Significance in Human Nasopharyngeal Carcinoma

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ABSTRACT

Purpose: p16 is an important inhibitor of cell cycle progression; absence of p16 can thus result in increased cellular proliferation. In nasopharyngeal carcinoma (NPC), absence of p16 has been reported in association with presence of the EBV and pRb. We therefore examined p16, pRb, and EBV-encoded RNA (EBER) expression in biopsy specimens from 84 patients with newly diagnosed NPC, who were treated primarily with curative radiation therapy. Our objective was to determine whether there was a correlation between these parameters and clinical outcome in NPC.

Experimental Design: Sections were cut from archival formalin-fixed, paraffin-embedded tumor blocks from NPC patients. p16 and pRb expression were determined using polyclonal and monoclonal antibodies, respectively. The presence of EBV was determined by in situ hybridization for EBER. The percentage of positively staining tumor nuclei was scored for p16 or pRb immunoreactivity. Relative intensity and proportion of cells with EBER signals were also documented.

Results: p16 expression was reduced (≤5% positive immunoreactivity) in 59 of 84 (70%) NPC samples; in contrast, pRb was observed in all (100%) tumors. EBER signals were detected in 67 of 83 (81%) NPC specimens. There was a weak correlation between EBER presence and loss of p16 (P = 0.1). Using a Cox regression model controlling for known prognostic parameters, such as age, weight loss, and tumor stage, complete absence of p16 expression (0%, i.e., no immunostaining identified throughout the specimen) was associated with an inferior overall survival rate (P = 0.022). In addition, EBER-positive NPC was strongly associated with improved overall survival (P = 0.005) as reported previously (Shi et al., Cancer, 94: 1997, 2002).

Conclusion: These results provide the first evidence suggesting that inactivation of p16 appears to be a significant predictor for poor overall survival in NPC. Given that reduced p16 expression is observed in the majority of patients with NPC, this indicates that therapeutic strategies targeting the p16 pathway may be a biologically rational approach for NPC. The favorable prognostic value of EBER suggests that future clinical trials with NPC should consider stratifying for EBER status.

INTRODUCTION

NPC is distinct from other epithelial malignancies in the head and neck region in that it affects a relatively young population and has a predilection for developing distant metastases (1). It has unique epidemiological characteristics, such as geographic distribution in Southeast Asia or the Mediterranean basin. In addition, its intimate association with the EBV is well established. Its anatomical location with close proximity to the skull base renders it a significant challenge for effective delivery of modalities, such as RT. With conventional RT, the overall 5-year survival rate hovers ~70% (2, 3), potentially related to inadequate coverage of the tumor volume (4). With the advent of intensity-modulated RT, the 4-year local control rate is superior, but the metastasis rate remains high at 40% (2, 5). The role of cis-platinum-based chemotherapy combined with RT remains controversial (3, 6–9), underscoring an urgent need to develop novel therapeutic strategies to improve outcome.

To design novel therapies rationally, a thorough understanding of the behavior of NPC is essential. Information de-
Loss of p16 Expression Has Prognostic Significance

Several groups have examined whether there are molecular pathology variables which can predict clinical outcome in NPC (13–17). Our group reported a strong association between the presence of EBV (as demonstrated by EBER signals) and improved outcome in NPC patients who were primarily treated with curative RT (11). EBV presence was also associated with Asian ethnicity, WHO type 2B tumors, and p53 overexpression. p53 overexpression also correlated with tumor microvessel density and tumor necrosis, although this bore no prognostic value in this cohort of patients.

The p16 tumor suppressor gene at 9p21 is an inhibitor of cell cycling by blocking the G1-S phase of the cell cycle (18). p16 operates through inhibition of cyclin-dependent kinase 4, which in turn inhibits phosphorylation of pRb to prevent progression of cells into the S phase (19). p16 is frequently deleted, mutated, or methylated in squamous cell carcinoma of the head and neck (20, 21), and this loss has predicted for poor clinical outcome in several human tumors (22, 23), presumably because of continued cancer cell proliferation. The importance of p16 in cancer development and progression has been recently reviewed (24).

p16 expression has been evaluated in NPC and is reported to be reduced in expression in 40–82% of cases (25–28). Two of these studies also demonstrated maintenance of pRb expression in all evaluable samples, despite the reduction in p16 immunostaining (25, 26). In these same two studies, EBER was detected in the majority of NPC samples (79–83%), which in one report, was associated with absent p16 expression (26), which may have a biochemical basis for this relationship (29).

The apparent paradox of a favorable prognostic marker (EBER), being associated with an unfavorable predictor (p16), is difficult to reconcile, based on information in the literature. In addition, none of these studies correlated any of these observations with clinical outcome.

Given these reports, and our previous experience (11), we evaluated a cohort of NPC patients who were primarily treated with RT from a single North American institution. Our hypothesis was that absence of p16 expression could be associated with EBV presence and that p16 may be a more significant prognostic variable; hence, absent p16 will correlate with a poor clinical outcome.

**MATERIALS AND METHODS**

**Patients.** This study is based on a cohort of 198 patients with NPC treated with curative intent between 1985 and 1992 at the Princess Margaret Hospital (3). Archival tumor blocks were available from 101 NPC patients, but the following were discarded: (a) 2 specimens because of incorrect histopathological diagnosis; (b) 11 because of inadequate amount of tissue remaining for immunohistochemistry; and (c) 4 because of heterogeneous immunohistochemical staining that could not be interpreted adequately. This series therefore consists of 84 patient specimens. The clinical, demographic, and tumor-related characteristics of the first 79 patients have been published previously (11). All 84 patients were treated with RT using a median dose of 66 Gy/33 fractions/6.5 weeks; only 4 patients received additional chemotherapy, using a neoadjuvant induction strategy. The majority of patients were male (61 or 73%), of Asian/Chinese descent (52 or 62%), had locally advanced disease (68 or 81% were Stage III or IV), and had undifferentiated WHO type 2B NPC (65 or 77%).

**Tissue Specimens.** All tumor specimens had been fixed previously in formalin and embedded in paraffin using routine methods. For each patient’s tumor, 4-μm sections were cut from one representative block for histological review and immunohistochemistry. The sections were stained with H&E and reviewed by a single pathologist (C. M.) to confirm the diagnosis of NPC. The tumors were classified in accordance to the WHO classification system (30). WHO type 1 is keratinizing squamous cell carcinoma, type 2A is nonkeratinizing differentiated carcinoma, and type 2B is nonkeratinizing undifferentiated carcinoma.

**Immunohistochemistry for p16 and pRb.** p16 and pRb immunoreactivity were evaluated using the Signet Kit (Signet Laboratories, Dedham, MA) with microwave antigen retrieval [citrate buffer (pH 6.0)]. The rabbit polyclonal antihuman p16 antibody (PharMingen, San Diego, CA) and mouse monoclonal antihuman pRb antibody (PharMingen) were used at dilutions of 1:500 (overnight) and 1:300 (for 1 h), respectively, at room temperature. Regions of nonneoplastic reactive cells surrounding NPC in the same section served as internal positive controls; negative controls were obtained by omitting the primary antibody. As an external positive control, the human NPC cell line (CNE-2Z) was used for positive p16 expression (31).

Immunostaining of p16 and pRb was evaluated using standard light microscopy. Cytoplasmic reactivity was disregarded as nonspecific, and only staining of tumor nuclei was scored as positive for either p16 or pRb immunoreactivity. A tumor was considered positive when the immunoscore was >5% for either p16 or pRb. Representative samples of p16 and pRb immunostaining are provided in Fig. 1. The percentage of positively stained tumor nuclei was then counted and recorded as 0, 1–5%, or >5%. Scoring for both molecular variables, on the entire set of 84 patient samples, was conducted by two examiners (A. A. M. and C. M.), who were blinded to the clinical outcome of the patients.

**Detection of Latent EBV (EBER) Infection.** The presence of EBER is commonly observed in undifferentiated NPC (15). In the current study, EBER was detected by using ISH. Details of this technique have been described in our previous work (11). Briefly, 4-μm sections were cut from paraffin-embedded blocks and mounted onto Superfrost/Plus slides (Fisher Scientific). After sections were predigested with proteinase K (1:30), a hybridization solution containing a fluorescein-conjugated EBV (EBER) peptide nucleic acid probe (DAKO, Mississauga, Canada) was applied for 2 h at 55°C. Alkaline phosphatase-conjugated anti fluorescein (DAKO) was then used to detect the hybridization products. 5-bromo-4-chloro-3-indolylphosphate nitroblue tetrazolium was applied as a chromogen. Slides were counterstained with or without hematoxylin. A positive hybridization signal was identified by dark brown staining. The scoring was graded as 0 (negative), 1 (<10% of...
Fig. 1 Immunohistochemical staining of p16 (A) and pRb (B) in NPC specimens using rabbit polyclonal antihuman anti-p16 antibody and mouse monoclonal anti-pRb antibody, respectively. A, note that p16 is negative in the tumor nucleus (arrow 1), whereas lymphocytes within and outside the tumor show positive nuclear staining for p16 (arrow 2). In B, there is strong immunostaining in tumor nuclei for pRb (arrow 3), with some lymphocyte nuclei also demonstrating positive pRb staining (arrow 4).
tumor nuclei being EBER positive), 2 (10–50% positivity), or 3 (>50% positivity) in accordance to the staining proportion and intensity.

**Statistical Analyses.** OS and DFS were defined from the time of diagnosis to the date of death or first failure, respectively, plotted using the Kaplan-Meier estimate (32). The significance of p16 and EBER presence was tested using a Cox proportional hazards model, as a sole variable in the model, as well as when adjusted for the clinical factors that were identified to be significant in the whole cohort of 198 patients. The significant clinical factors were age, stage, and weight loss for both OS and DFS. EBER was dichotomized as 0 versus 1, 2, or 3. Associations between p16, EBER, and the clinical factors were tested using Fisher’s exact test. Because pRb was positive in every specimen, this precluded the usefulness of pRb as a distinguishing parameter.

**RESULTS**

Clinical Description. With a median follow-up time of 8.4 years (range from 3.7 to 13.2 years), the 5-year OS for the 84 patients was 71%, the DFS rate was 56%, and the local control rate was 68% (Fig. 2). The outcome for this subgroup of 84 patients completely overlaps that of the larger cohort of 198 patients (3).

**p16 and pRb Expression in NPC.** Cytoplasmic immunoreactivity was heterogeneous; in some cases, a nonspecific cytoplasmic staining obscured the interpretation of the nuclear staining so that these cases were excluded from the study. The clinical and molecular characteristics of the entire cohort are outlined in Table 1. Positive p16 expression (defined as >5% positively staining tumor nuclei) was observed in 25 (30%) of these NPC specimens; 59 (70%) specimens had ≤5% of tumor nuclei immunostaining for p16. Among these 59 samples with reduced p16 expression, immunoexpression was completely absent in 35 specimens (42% of all samples, as in Table 1). For the remainder of this study, “reduced” expression refers to ≤5% immunoexpression for p16; “absent” p16 refers to 0% p16 expression. There was strong positive nuclear staining in lymphocytes, fibroblasts, normal surface mucosal, and squamous respiratory epithelium and seromucous glands, providing internal positive controls (Fig. 1A). The proportion of specimens demonstrating reduced p16 staining in the WHO 2A and 2B groups was 42 of 71 (59%) versus 7 of 13 (54%) in the WHO

![Fig. 2](image-url) A Kaplan-Meier plot for survival on the 84 patients constituting the study population. The median follow-up was 8.4 years. The 5-year OS was 71%; the 5-year DFS was 56%.

### Table 1 Clinical and molecular characteristics of patients

<table>
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<td>Age &gt;50</td>
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<td>EBER 2</td>
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1 group (Fisher's exact test; \( P = 0.77 \)). pRb immunostaining was positive (defined as \( \geq 5\% \) positively staining tumor nuclei) in all 84 cases (Fig. 1B). In these cases, the majority demonstrated \( > 75\% \) immunoexpression in the nuclei of these specimens.

**EBER.** The majority of NPC tumors was EBER positive (67 of 83 or 81%); EBER was completely absent in 16 cases (19%). In 1 sample, no tumor cells were available for EBER determination. There were 5 specimens that were both EBER negative and p16 negative. In EBER-positive specimens, ISH demonstrated nuclear signals only in the cancer cells, none in the normal tissues (11).

There was a statistical trend in the association between reduced p16 expression and positive EBV status, as defined by positive EBER signals using ISH. p16 was reduced (\( \leq 5\% \) of cells with p16 immunostaining) in 24 of 28 (86%) of the highly EBER expressed (score of 3) tumors. In contrast, p16 was reduced in only 34 of 55 (62%) lower EBER expressed samples (score 0, 1, 2; \( P = 0.04 \)). Similarly, p16 was reduced in 15 of 28 (54%) in the highly expressed EBER samples versus 19 of 55 (35%) in the lower EBER expressed samples (\( P = 0.1 \)).

**Molecular Pathology Variables and Clinical Outcome.**

On univariate analysis (Fig. 3), p16 status was observed to be nonsignificant for OS and DFS (\( P = 0.2 \) and 0.8, respectively). Reduced p16 (i.e., \( \leq 5\% \) of the cells with immunoreactivity) was also nonsignificant when adjusted for the aforementioned clinical factors (age, weight loss, and stage of disease). However, when absent p16 (i.e., complete absence of p16 immunoreactivity) was adjusted for age, weight loss, and stage, this was observed to be significantly associated with poor OS (\( P = 0.02 \)).

EBER status predicted for both improved OS and DFS rates (\( P = 0.0003 \) and 0.023, respectively) in univariate analysis. Fig. 4 demonstrates the effect of EBER on survival. The 5-year OS for the group of patients with EBER-negative tumors was 42% compared with 79% for the group of patients with EBER-positive tumors (\( P = 0.0003 \)). Likewise, the 5-year DFS for patients with EBER-negative tumors was 31% compared with 63% for patients with EBER-positive tumors (\( P = 0.02 \)). EBER was also significantly correlated with OS when controlled for the clinical prognostic factors (\( P = 0.005 \)). The hazard ratio for the effect of EBER on survival when controlled for clinical factors was 0.3.

Given the significance of both p16 and EBER status on survival, we proceeded to examine for a possible interaction between p16 and EBER with survival. Our analysis indicated that absent EBV status confers a poor OS, regardless of p16 expression. In the EBER-negative group of patients, the 5-year OS ranged from 30 to 45% (Fig. 5). However, if the tumor was EBER positive, absence of p16 can further distinguish outcome. Specifically, the best prognostic group was tumors which were both EBER and p16 positive (5-year OS of 89%) versus EBER positive but absence of p16 (OS of 66%).

**DISCUSSION**

This is the first observation of a correlation between p16 and EBER expression with clinical outcome, in that absence of p16 was associated with poor OS in this cohort of NPC patients. Age, weight loss, and stage were already established to be independent variables predicting outcome in the analysis of 198 NPC patients (3). When p16 expression was analyzed controlling for these three variables in a multivariate analysis, an association between absence of p16 expression and worse survival was observed (\( P = 0.02 \)). However, in univariate analysis, absence of p16 expression was not significant, indicating that the effect of p16 is confounded by the clinical factors.

In our current cohort, 70% of the specimens showed reduced p16 immunostaining (\( \leq 5\% \)), which is consistent with the range from 40 to 82% reported elsewhere (25–28). Shibosawa et al. (26) noted a significant association between absent p16 expression with the presence of EBER, which is similar to our
own data of increasing intensity of EBER signals being associated with reduced p16 expression in NPC biopsy samples.

The above data suggest that loss of p16 expression may be involved in NPC development or progression, but the precise mechanism remains to be elucidated. Hypermethylation, deletion, or mutation of p16 have been observed as potential mechanisms of p16 deregulation in many tumors (33). The observation of EBER positivity correlating with absence of p16 might be attributable to LMP-1. LMP-1 is one of the gene products of the EBV, and there is biochemical evidence indicating that LMP-1 can silence the p16 promoter through methylation (29). Hence, one possible scenario for NPC development or progression may be related to LMP-1 inactivating p16 through promoter methylation, resulting in increased tumor cell proliferation, ultimately leading to reduced OS.

The finding that pRb was immunohistochemically detected in all NPC specimens in our study is similar to reports from other groups (25, 26). This constant pRb expression may not provide useful information with regards to pRb activity, because the pRb antibody does not distinguish the various phosphorylated forms of pRb, which confer differential functional status (25).

The link between positive EBER status and improved OS and DFS for NPC has been reported previously by our group (11). The present study is based on the same patient cohort but is strengthened by the addition of new cases, longer follow-up,

Fig. 4 A Kaplan-Meier plot for OS as a function of EBER status (absent versus present), for the 84 patients in this study. The 5-year OS for the two groups were 42 and 79%, respectively ($P = 0.0003$).

Fig. 5 A Kaplan-Meier plot for OS as a function of combined p16 and EBER status, for the 84 patients in this study. The 5-year OS for EBER+/p16+, EBER+/p16−, EBER−/p16+, and EBER−/p16− were 89, 66, 45, and 30%, respectively.
and the p16 data. The median follow-up on this current cohort of patients has lengthened to 8.4 years, and with further maturation of the clinical data, the relationship between EBER positivity and improved outcome is further substantiated for both OS and DFS (P = 0.0003 and 0.02, respectively).

Our observation of EBER positivity, as a surrogate for EBV presence, and its association with an improved outcome in NPC could have significant implications in the management of these patients. Marks et al. (34) have documented an improved 5-year relative survival of 65% for nonkeratinizing and undifferentiated NPC patients versus 37% for keratinizing NPC patients. However, they did not have information on the EBV status of these patients’ tumors. Our observation suggests that their data might be explained by the presence of EBV in the patients with the superior outcome. It may be that EBV-positive and -negative NPC are actually two distinct clinical and biological entities; hence, they might be managed differently.

This issue of concurrent chemotherapy in the management of NPC in benefiting WHO type 2B (i.e., EBER positive) as opposed to the more typical NPC histologies in the United States was discussed recently (3, 6, 12). In our recent retrospective analysis of 198 NPC patients primarily treated with radical RT alone (3), we compared their outcome to that of the participants in the Intergroup 0099 trial (6). In this randomized trial of RT alone versus RT plus chemotherapy, the outcome of the Intergroup RT alone patients was inferior to that of our similarly staged patients, also treated with RT alone. One possible biological explanation for the superior outcome for our Princess Margaret Hospital cohort might be attributable to the majority (75–81%) of our patients having EBV-positive NPC. Hence, we would suggest that EBER status should be considered as a stratification variable for future clinical trials involving NPC patients.

This recommendation may be strengthened by a recent report on NPC patients treated from a single American institution, whereby there was a difference in pattern of failure between Chinese versus non-Chinese patients (12). Intriguingly, Chinese patients had a greater propensity for developing distant metastases, although no significant survival difference was observed. However, there was a statistically significant difference in OS as a function of histology, with “lymphoepithelioma” patient faring better than other histologies. Given the correlation between “lymphoepithelioma,” or WHO type 2B with EBER status (11, 35–37), this further supports the contention that EBV-positive tumors have a superior outcome to EBV-negative NPCs.

We4 and others (38) have preclinical data indicating that p16 gene therapy in combination with RT is particularly effective against NPC cells which lack p16 expression. Given that the majority of NPC loses p16 expression, and its absence may confer a worse outcome, therapeutic strategies targeting the p16 pathway could be an effective approach. In summary, two conclusions can be drawn from this current analysis: (a) we confirm that p16 expression is reduced or absent in the majority of patients with NPC, and this appears to be a predictor for worse survival; and (b) with more mature follow-up, we consolidated our previous observation of EBER positivity being associated with improved survival for NPC patients, suggesting that EBER-positive and -negative NPC are distinct clinical entities. Hence, we would recommend that for future clinical trials for NPC patients, EBER status should be considered as a stratification variable.

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