Nasopharyngeal carcinoma (NPC) is a malignant epithelial carcinoma of the head and neck site, with incidences of 20 to 30 per 100,000 people in areas such as Southeast Asia (a region of >100 million people) and the Mediterranean basin (a region of >300 million people) (1). NPC is anatomically located in close proximity to the base of the skull, rendering it a significant challenge for conventional and experimental treatments. In fact, inadequate tumor volume coverage may be partially responsible for the modest 5-year overall survival (OS) rate of ~70% when patients are treated with conventional radiotherapy alone (2–4). Although intensity-modulated radiotherapy does improve local control, the risk of distant metastases remains high at ~40% (2). A predilection for developing distant metastases, a young median age of presentation (at ~50 years), and the intimate association with EBV are features that render NPC a unique head and neck epithelial malignancy (3). The latent presence of EBV is unique to malignancies such as NPC, Burkitt’s lymphoma, Hodgkin’s lymphoma, peripheral T cell lymphomas, natural killer cell lymphomas, and gastric carcinomas (1). Approximately 75% to 81% of patients with NPC worldwide harbor the EBV genome, which is present in the type II latency. This form of latency is characterized by the expression of a limited set of viral genes, including \( \text{EBNA1}, \text{LMP1}, \text{LMP2} \), and \( \text{EBER} \) (1). These genes almost certainly contribute to tumor development. EBNA1 for example, binds and inhibits USP7/HAUSP. USP7 normally deubiquitinates the tumor suppressor p53, thereby stabilizing it and facilitating p53-mediated growth suppression and apoptosis (6). Therefore, the presence of EBNA1 could compromise the normal function of p53. LMP1 has been shown to up-regulate Bcl-2 and antiapoptotic Bcl-2 family members, possibly through nuclear factor \( \kappa B \) (1). LMP1 also induces the overexpression of survivin (7). Survivin has been
implicated in both cell death inhibition and mitotic regulation (8), by binding to the X-linked inhibitor of apoptosis in the cytoplasm to synergistically inhibit apoptosis (9), and by associating with the mitotic apparatus. EBV-induced up-regulation of survivin leads to increased cell proliferation (7), whereas inhibition of survivin results in catastrophic defects in mitosis, failed cytokinesis, and polyplody (8). The significance of p53, Bcl-2, and survivin in EBV-negative NPC remains undetermined.

p53, Bcl-2, and survivin are dysregulated in other head and neck cancers. p53, for example, is associated with poor patient outcome in laryngeal squamous cell carcinomas and oral premalignant lesions (10). Bcl-2 is up-regulated in head and neck squamous cell carcinomas, and inhibits chemotherapy-induced apoptosis in these tumors (11, 12). Survivin is also up-regulated in head and neck squamous cell carcinomas, and has been associated with poor survival in esophageal cancer (11, 13).

Given the above reports, we evaluated a cohort of patients with NPC who were primarily treated with radiation therapy from a single North American institution. Our objective was to determine the prognostic significance of key molecular factors, specifically EBV, p53, Bcl-2, and survivin, in NPC.

### Materials and Methods

**Patients.** Between the period of 1985 and 1992, 198 patients with NPC were treated with curative intent at the Princess Margaret Hospital (3). Archival formalin-fixed and paraffin-embedded tumors were obtained from 80 of these patients prior to treatment. The clinical characteristics of this subgroup of patients have been previously published (14, 15). As shown in Table 1, the majority of patients were male (60 of 80, or 75%), of Asian/Chinese ethnicity (49 of 80, or 61%), had locally advanced stage III or IV disease (66 of 80, or 83%), and had undifferentiated WHO type 2B NPC (60 of 80, or 75%). All patients were treated with curative intent. The median radiotherapy dose was 66 Gy/33 fractions/6.5 weeks delivered using a parallel pair opposed field technique. Only four patients received additional chemotherapy, delivered in a neoadjuvant fashion.

**Tissue specimens.** For each patient’s tumor block, 4-μm sections were cut and mounted onto microscopy slides. One representative section from each block was stained with H&E (Fig. 1A), and then reviewed by a single pathologist (C. MacMillan) to confirm the diagnosis and WHO classification. Keratinizing squamous cell carcinoma is classified as WHO type 1, nonkeratinizing differentiated carcinoma as type 2A, and nonkeratinizing undifferentiated carcinoma as type 2B (also known as type 3) NPC (5). Due to the small size of these biopsy specimens, not every patient had sufficient remaining tumor tissues for all these variables to be evaluated.

**Detection of latent EBV infection.** The latent presence of EBV in NPC biopsies was detected using EBER *in situ* hybridization as previously described (14, 15). Briefly, sections were predigested with proteinase K for 10 minutes at 37°C, probed with a fluorescein-conjugated EBV (EBER) peptide nucleic acid probe (DAKO, Mississauga, Ontario, Canada) for 2 hours at 55°C, and detected with an alkaline phosphatase–conjugated anti–fluorescein antibody (DAKO). 5-Bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium was used as a chromogen, and several slides were counterstained with a light hematoxylin stain. Dark brown staining was identified as the positive hybridization signal. Scoring was defined as follows: 0 (negative), 1 (<10% positive tumor nuclei), 2 (10-50% positive tumor nuclei), or 3 (>50% positive tumor nuclei). Tumors that were scored 1, 2, or 3 were considered EBER-positive.

**Immunohistochemistry for p53, Bcl-2, and survivin.** For immunohistochemistry, microwave antigen retrieval was used in combination with the Level-2 Ultra Streptavidin (horseradish peroxidase) system (Signet Laboratories, Dedham, MA). Detection of p53, Bcl-2, and survivin utilized the mouse monoclonal antihuman p53 (clone PAb 1801, 1:100 dilution; Novocastra Laboratories, Newcastle upon Tyne, England), mouse monoclonal antihuman Bcl-2 oncoprotein (clone 124, 1:50 dilution; DakoCytomation, Carpinteria, CA), and rabbit polyclonal antisurvivin (NB 500-201, Lot 9, 1:50 dilution; Novus Biologicals, Littleton, CO) antibodies, respectively.

In order to determine the percentage of positive tumor cells, light microscopy was used to count at least 300 tumor cells in the three most densely staining fields (>400). For p53, a tumor was considered positive when ≥10% of tumor nuclei expressed p53. For Bcl-2, a tumor was considered positive when >0% of tumor cells expressed Bcl-2. For survivin, a tumor was considered positive for nuclear survivin when ≥5% of tumor nuclei expressed the protein, and highly positive for nuclear survivin when >25% of tumor nuclei expressed the protein. A tumor was considered positive for cytoplasmic survivin when >0% of tumor cells expressed the cytoplasmic protein.

### Statistical analyses

OS was defined as the time of diagnosis to date of death; disease-free survival (DFS) was defined as the time of diagnosis to date of first failure. The OS and DFS estimates over time were calculated using the Kaplan-Meier method (16). Associations between EBER, p53, Bcl-2, or survivin with OS or DFS were analyzed using the log-rank test. Correlations between EBER and p53, Bcl-2, or survivin were analyzed using χ² test, as were correlations between the molecular factors with ethnicity and WHO.

### Table 1. Clinical and molecular characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
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</tr>
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<tbody>
<tr>
<td>Age (y)</td>
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</tr>
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<td>≤50</td>
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<tr>
<td>&gt;50</td>
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<tr>
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<tr>
<td>Chinese</td>
<td>43</td>
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<td>Black</td>
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<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14</td>
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<tr>
<td>II</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>37</td>
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<tr>
<td>IV</td>
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<td>WHO classification</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>2A</td>
<td>0</td>
</tr>
<tr>
<td>2B</td>
<td>60</td>
</tr>
<tr>
<td>EBER</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>2</td>
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<td>1</td>
<td>14</td>
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<td>2</td>
<td>16</td>
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<tr>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>p53 (%)</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>46</td>
</tr>
<tr>
<td>≥10</td>
<td>34</td>
</tr>
<tr>
<td>Bcl-2 (%)</td>
<td></td>
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<tr>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>&gt;0</td>
<td>48</td>
</tr>
<tr>
<td>Nuclear survivin (%)</td>
<td></td>
</tr>
<tr>
<td>0 to &lt;5</td>
<td>32</td>
</tr>
<tr>
<td>5-25</td>
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<tr>
<td>&gt;25</td>
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Results

Expression of molecular markers in NPC. As shown in Table 1, the majority of these patients’ tumors are EBV-positive, whereby EBER in situ hybridization signal intensity (Fig. 1B) was detected in 64 of 78 (82%) of these biopsies (in two samples, EBER could not be done due to insufficient tumor tissues). These EBER-positive NPC’s were associated with ethnicity, in that more Asian/Chinese patients (45 of 48, or 94%) had EBER-positive tumors than African/Caucasian patients (19 of 30, or 63%; \( P = 0.0007 \)). In addition, the latent presence of EBV was more common in WHO type 2 (56 of 65, or 86%), than in WHO type 1 (8 of 13 or 61%) NPC (\( P = 0.04 \)).

Overexpression of p53, defined by nuclear immunostaining for p53 in \( \geq 10\% \) of tumor nuclei, was observed in 34 of 80 (43%) of NPC specimens (Table 1; Fig. 1C). p53 overexpression was associated with EBV in that only 1 of 14 (7%) of EBER-negative samples overexpressed p53 (Table 1; Fig. 2A). In contrast, 33 of 64 (52%) of the EBER-positive NPC biopsies also overexpressed p53 (Table 1; Fig. 2A), which is statistically significant (\( P = 0.002 \)). Similarly, more Asian/Chinese patients (27 of 49, or 55%) had p53 overexpressing tumors than African/Caucasian patients (7 of 31, or 23%; \( P = 0.004 \)).

Similar to p53, Bcl-2 expression (Fig. 1D) was associated with the presence of EBV in that 5 of 14 (36%) EBER-negative biopsies expressed Bcl-2 (Table 1; Fig. 2B). In contrast, 42 of 64 (66%) EBER-positive tumors were also Bcl-2-positive (\( P = 0.04 \); Table 1; Fig. 2B). Bcl-2 expression was more commonly observed in Asian/Chinese patients (33 of 49, or 67%), as opposed to a lower frequency in African/Caucasian patients (15 of 31, or 48%), although the latter association was not statistically significant (\( P = 0.09 \)).

Both nuclear and cytoplasmic survivin expressions were assessed using immunohistochemistry (Fig. 1E and F). Expression of nuclear survivin (defined as \( \geq 5\% \) tumor nuclei immunostaining for survivin) was associated with EBV-positivity in that only 5 of 14 (36%) of EBER-negative NPC biopsies had nuclear survivin expression (Table 1; Fig. 2C). In contrast, 43 of 64 (67%) EBER-positive tumors also expressed nuclear survivin (Table 1; Fig. 2C; \( P = 0.03 \)). Not surprisingly, nuclear survivin expression correlated strongly with cytoplasmic survivin in that only 13 of 36 (36%) NPC biopsies with no cytoplasmic survivin had nuclear survivin expression. In contrast, 35 of 44 (80%) NPC biopsies expressed both cytoplasmic and nuclear survivin (\( P < 0.0001 \)). NPC biopsies from Asian/Chinese patients were more commonly nuclear survivin-positive (35 of 49, or 71%) than biopsies from African/Caucasian patients (13 of 31, or 42%; \( P = 0.009 \)).

Prognostic value of molecular variables. The median follow-up time for this group of patients has now reached 11.4 years, with 10-year OS and DFS of 63% and 50%, respectively. Similar to our previous observations (14, 15), EBER-positive patients continue to experience a statistically significant better OS compared with EBER-negative patients (Fig. 3A). Specifically, the 10-year OS rates were 68% versus 48% for the
EBER-positive and -negative groups of patients, respectively ($P = 0.03$). Interestingly, the 10-year DFS rates were also superior in the EBER-positive group (54% versus 43%), but this relationship was no longer statistically significant ($P = 0.2$; Fig. 3B).

Neither p53 nor Bcl-2 expression provided any prognostic value for either OS or DFS for this group of patients. However, nuclear survivin had an intriguing “polynomial” relationship in that the OS hazard was lowest when the percentage of tumor nuclei expressing survivin was 10%, but this hazard increases as the percentage decreases from 10% to 0%, or when the percentage increases from 10% to 80% (Fig. 4A). This relationship is reflected in the actuarial survival graph (Fig. 4B), which shows that OS was superior when the percentage of tumor nuclei expressing survivin was in the intermediate group ($\geq 5\%$ to $\leq 25\%$) versus the low ($< 5\%$) or high ($> 25\%$) expressors (Table 2). For these two disparate groups, the 5- and 10-year actuarial OS rates were 84% versus 65% and 74% versus 53%, respectively ($P = 0.05$).

Discussion

We have previously reported the largest North American series of NPC samples from a single institution subjected to detailed molecular pathology analyses (14, 15). Our previous observations noted the strong association of positive EBER status with superior OS and DFS (14), and the negative prognostic indicator of absent p16 expression in NPC (15). Our current study uses a similar population of patients, but now with an extended follow-up time of 11.4 years (versus 4.8 years [14] and 8.4 years [15] for the previous studies). In this current study, we continue to observe that patients with EBV-positive NPC experience better OS than EBV-negative NPC. The difference in DFS rates is no longer observed with the longer follow-up time (Fig. 3B). (Similar observations were recently reported on 2,687 patients with NPC treated in Hong Kong [17], wherein the DFS curves never plateau, indicating that patients with EBV-positive NPC continue to relapse, even several years after the completion of treatment).

EBV-positive tumors expressed p53, Bcl-2, and nuclear survivin. EBV-negative tumors, on the other hand, tend not to express p53, Bcl-2, or survivin. Similar to other reports, EBV-positive NPC tumors are associated with both ethnicity (Asian/Chinese versus Africans/Caucasians), and WHO sub-type 2A and 2B (versus type 1 NPC; ref. 18). Most significant, however, is the first demonstration of a polynomial prognostic factor whereby intermediate nuclear survivin expression is associated with better OS compared to patients with a low or high proportion of survivin immunostaining.

Our observation that WHO type 1 NPC is more commonly EBV-negative, which in turn, is associated with reduced OS (Fig. 3A) is corroborated by a large American population–based study, whereby the outcome for 5,069 patients with NPC was significantly associated with histology (19). WHO
type 1 NPC had a poorer outcome with a 5-year OS rate of 37%, as opposed to 65% for patients with either type 2A or 2B histologies (19). As we have stated previously (15), we believe that EBER-positive and EBER-negative NPC are two distinct clinical and biological entities, hence, EBER status should be used as a stratification variable for future NPC clinical trials.

These clinical data are borne out by biology, whereby the protein expression profile of EBV-positive tumors, which overexpress p53, Bcl-2, and nuclear survivin, are distinct from that of EBV-negative tumors (Fig. 2). Other groups have also reported a significant association between p53 and Bcl-2 expression in NPC (20, 21), along with an association between EBV with both p53 and Bcl-2 overexpression in adult (22) and pediatric (23) NPC. Thus, one would surmise that the optimal treatment modalities or regimens for EBV-negative and EBV-positive NPC should be different, although we might require additional insights from future gene expression microarray studies to fully elucidate the distinct molecular profiles between EBV-positive and EBV-negative NPC.

The association between p53 overexpression and EBV status is reproducibly reported (24, 25), but its underlying mechanism is complex. The p53 gene is rarely mutated in primary NPC (26), providing evidence that latent EBV genes are likely responsible for alterations in p53 expression. The EBV protein EBNA1 binds USP7, thereby preventing deubiquitination of p53 (6). Ablation of USP7, however, has recently been shown to result in p53 accumulation (27). This might be explained by USP7 stabilizing Mdm2, thereby preventing Mdm2-mediated p53 degradation (27). The mechanism of EBV-induced p53 overexpression is further complicated by other EBV gene products such as BZLF1 and EBNA5, which can also bind p53 (28, 29). In addition, EBNA2 can also promote p53 phosphorylation and induce p21 expression (30). Hence, it is likely that multiple EBV players interact with p53, ultimately resulting in its nuclear accumulation, and probable compromised function.

Bcl-2 overexpression has been reported in ~80% of NPC cases, and is also associated with EBV (31). The EBV oncogenic protein, LMP1, can directly induce Bcl-2 expression (1). Bcl-2-targeted experimental molecular therapies for NPC, such as BimS gene therapy (32), or Bcl-2 antisense therapy (33), have been shown to be highly effective, and hence, would be predicted to have a greater benefit for patients with EBV-positive NPC.

One of the most intriguing observations in this study relates to the poor clinical outcome when the proportion of nuclear survivin–expressing tumor cells is either too low or too high. Survivin itself seems to be a multifunctional protein, playing important roles in both mitotic regulation and apoptosis inhibition (8). In terminally differentiated normal tissues, survivin expression is undetectable, but survivin overexpression has been observed in numerous human malignancies (8). Thus, there has been substantial interest in survivin as a therapeutic target. Recent evidence suggests that survivin gene transcription is linked to mitotic progression, and because cancer cells are globally deregulated in cell cycling, survivin is overexpressed during all phases of the cell cycle (8). Survivin can be directly up-regulated via LMP1 (7) and the WNT-β-catenin signaling pathway (34), which is known to be activated in NPC from microarray expression studies (35). Of note, wild-type p53 can transcriptionally repress survivin (8), therefore, the dysfunction of p53 in NPC (mediated via EBV proteins) might also contribute to survivin up-regulation because p53 itself is rarely mutated in NPC (26).

The inhibition of survivin can lead to aberrant mitotic progression, with supernumerary centrosomes, multipolar mitotic spindles, failed cytokinesis, multinucleation, premature sister-chromatid separation, dysregulation of spindle-checkpoint activation, and/or apoptosis (8). Therefore, although very low survivin levels would normally prevent functional cell division, the combination of low survivin with resistance to cell death (mediated by the overexpression of antiapoptotic proteins such as Bcl-2) in NPC may contribute to genomic instability.

Conversely, increased survivin levels can preserve spindle function against microtubule poisons, promoting resistance to chemotherapy (8), and promoting cell proliferation (7). Similarly, high cytoplasmic survivin levels (which is associated with high nuclear survivin expression) could lead to the inhibition of proapoptotic caspases (8, 9). Interestingly, a recent study showed that absent caspase-3 activation predicted for rapid fatality in patients with NPC (36).

The literature regarding the prognostic value of survivin is currently confusing. Some articles report that increased nuclear survivin predicts for poor outcome (37, 38), or improved outcome (39, 40), or has no effect (41, 42). Some of this controversy has been attributed to technical reasons (43), but we submit that perhaps another reason for the inconsistencies reported in the literature might be attributed to this unusual polynomial relationship of nuclear survivin with biological and clinical outcome. Most investigators in the prognostic field of research think of prognostic variables as binary, dichotomized, or even continuous variables, but in a linear fashion. We propose a paradigm shift, offering nuclear survivin as a variable whereby both too high and too low a proportion of survivin-expressing tumor cells is associated with biological aggressiveness, translating to poorer clinical outcome. A similar phenomenon has been encountered in bladder cancer, in which both elevated and absent pRb expression is associated with lower survival rates (44). Other underlying factors may be responsible for these nonlinear observations; for example, elevated pRb is associated with loss of p16 in bladder cancers (45).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazards ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear survivin alone</td>
<td>2.04 (0.98-4.24)</td>
<td>0.06</td>
</tr>
<tr>
<td>Combined model</td>
<td>2.04 (0.98-4.24)</td>
<td>0.06</td>
</tr>
<tr>
<td>Age</td>
<td>1.05 (1.02-1.08)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Stage</td>
<td>14.1 (1.9 to &gt;30)</td>
<td>0.01</td>
</tr>
<tr>
<td>Nuclear survivin with age and stage</td>
<td>2.3 (1.1-4.9)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE: Results of the Cox regression model incorporating age (at time of diagnosis), and stage dichotomized (I/II versus III/IV), with nuclear survivin dichotomized as either (α) <5% or >25% versus (β) >5% to ≤25%.
The dual nature of nuclear survivin is not an uncommon biological phenomenon; many other proteins possess the ability to function as both tumor promoters and tumor suppressors. Members of the claudin family, such as claudin-4, may increase or decrease invasiveness depending on the molecular circuitry of the cell (46). Nucleophosmin inactivation leads to unrestricted centrosome duplication and genomic instability (47), but nucleophosmin overexpression leads to IRF-1 (antioncogenic transcription factor) inhibition and can result in malignant transformation (48). In addition, oncogenes such as Myc and nuclear factor-$\kappa$B may induce apoptosis, the latter being sometimes required for p53-mediated cell death (49, 50).

This novel observation would obviously require corroboration by other groups, and in other human malignancies. The total number of patients in each of the three subgroups is somewhat limited, hence, the data are not that robust. In addition, this specific polynomial observation was not part of our original hypothesis, but instead, was a data-driven evaluation.

In conclusion, this study confirms the effect of EBV status on OS, and documents that p53, Bcl-2, and nuclear survivin are overexpressed proteins in EBV-positive NPC. The potentially dual nature of survivin, which predicts for poor patient outcome at either high or low proportional expression, has been observed for the first time, in a clinical setting.

Acknowledgments

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Clinical Cancer Research

Prognostic Significance of the Epstein-Barr Virus, p53, Bcl-2, and Survivin in Nasopharyngeal Cancer

Kenneth W. Yip, Wei Shi, Melania Pintilie, et al.


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