Significance of c-Myc and Bcl-2 Protein Expression in Nasopharyngeal Carcinoma

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Objective: To investigate the relationship between oncogene protein expression and nasopharyngeal carcinoma outcome.

Design: Tumor samples from 51 patients with nasopharyngeal carcinoma were analyzed by immunohistochemical staining for expression of Bcl-2 and c-Myc protein before irradiation. Five-year follow-up data were available.

Results: Thirty-one (61%) of 51 tumors expressed Bcl-2 protein, and 29 (57%) of 51 tumors expressed c-Myc protein. In the Bcl-2+ group, 20 (65%) and 19 (61%) of 31 patients experienced recurrence or died, respectively, whereas only 7 (35%) and 5 (25%) of 20 patients with Bcl-2− tumors did so. On the other hand, patients with c-Myc+ tumors had lower recurrence and death rates (38% [11/29] and 34% [10/29], respectively) than those with c-Myc− tumors (73% [16/22] and 64% [14/22], respectively). A statistically significant association was confirmed between Bcl-2 and c-Myc positivity and patient outcome.

Conclusion: Expression of Bcl-2 and c-Myc protein seems to be a useful marker to reflect irradiation response and to predict illness condition and patient outcome.


Despite great advances in therapy, the survival rate of patients with nasopharyngeal carcinoma (NPC) has not substantially improved. Radiotherapy is considered standard treatment for NPC, and responders to irradiation have a better survival rate than nonresponders. The survival rate could be better if we could predict patients’ irradiation response and prognosis. The search for reliable markers to predict therapy response and prognosis is critical because, if available, the markers could help identify patients for whom intensive adjuvant therapy is worthwhile. Recently, the focus has turned to the expression of proto-oncogenes as potential prognostic factors.

Radiation induces its therapeutic effect via at least 2 characteristically different modes of cell death, currently termed necrosis and apoptosis. It has been reported that the level of spontaneous apoptosis before therapy predicts tumor responsiveness to irradiation in uterine cervical carcinoma. Some oncogenes, such as bcl-2, have a role in the apoptosis pathway; therefore, Bcl-2 protein expression may be of potential importance in the modulation of responsiveness to irradiation.

Bcl-2 is an intracellular membrane protein capable of blocking programmed cell death induced by several stimuli, preventing tumor cells from dying in response to severe DNA damage from radiotherapy. Moreover, neoplastic cells exhibit disturbances in the process of programmed cell death and are less likely to be responsive to treatment with chemotherapeutic agents and irradiation.

Results of several studies indicate that genetic alterations in the c-myc oncogene play an important role in the induction and progression of human cancer. The occurrence of c-myc oncogene amplification in breast cancer has been related to a poor prognosis. The product of the c-myc proto-oncogene is c-Myc protein, and overexpression of c-Myc protein in breast cancer to some extent correlates with gene amplification. c-Myc protein exerts diverse effects on cell behavior. Data gathered to date indicate that c-Myc protein plays a critical role in normal progression of the cell cycle, in inhibition of terminal differentiation, and in induction of programmed cell death.

However, no attempt was made in any of these studies to investigate Bcl-2 and c-Myc protein expression in NPC simul-
taneously or to correlate their expression with the subsequent occurrence. In the present study, we examined staining of the 2 oncoproteins in cancer tissue simultaneously before therapy; 5-year follow-up data were available. We conducted a prospective study to determine whether there is any relationship between their expression and the histologic grade and also patient outcomes to elucidate the potential role of oncoproteins as prognostic markers in NPC.

**METHODS**

**ANALYSIS OF PATIENTS**

Fifty-three patients with nasopharyngeal squamous cell carcinoma were originally included in this study (31 men and 22 women; mean age, 48 years). The patients were free of metastasis (as determined by computed tomography in some patients and by magnetic resonance imaging in others), and they received radiotherapy until clinical healing (cobalt Co 60: 6000-8000 rad [60-80 Gy] in 6-8 weeks). One patient was removed from therapy midway because of a serious adverse response to irradiation, and 1 patient was unable to be contacted for follow-up; these patients were not included in the study. Five-year follow-up data were available for 51 cases.

**IMMUNOHISTOCHEMICAL STAINING**

Specimens were obtained before therapy, fixed in 10% neutral buffered formalin, and embedded in paraffin. Serial 5-mm-thick sections were obtained from each specimen for hematoxylin-eosin and immunohistochemical staining. Slides were deparaffinized in xylene, rehydrated in graded alcohol, and incubated in 0.3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by 10-minute enzymatic digestion of tissue with 0.1% trypsin/EDTA. Nonspecific binding sites were blocked by 20-minute incubation with nonimmune horse serum.

The primary antibody used for detection of Bcl-2 was a monoclonal mouse IgG (Dako Co Ltd, Kyoto, Japan). With a working dilution of 1:100, tissue samples were incubated for 2 hours at room temperature. Staining was completed using an avidin-biotin-peroxidase complex method after 30-minute incubation with a secondary antibody. Color development was achieved using chromogen 3,3′-diaminobenzidine.

Immunostaining for c-Myc was performed using a cat monoclonal IgG (Dako Co Ltd). The antibodies recognized active forms of the proteins. Incubation of samples for 2 hours at room temperature with primary antibody at a working dilution of 1:50 was followed by incubation with a secondary antibody for 30 minutes. Staining was completed as described for Bcl-2. Positive and negative controls were provided on each series of tissue blocks studied. Positive controls comprised normal human skin samples obtained during surgical procedures for Bcl-2 and c-Myc. Negative controls were included by using trp E IgG as suggested by the manufacturer as a useful negative control that does not react with mammalian proteins. All negative controls were uniformly devoid of any staining, and strong positivity was seen in positive control specimens.

Expression was scored semiquantitatively by 2 observers analyzing 5 to 10 higher-power fields under an optical microscope (original magnification ×40). The results were quantified as negative (0) if no staining was observed in any cell and as positive if the neoplastic cells exhibited staining in 1% to 9% of the cells (+), 10% to 50% (++), and more than 50% (+++). Examples of positive and negative reactions are shown in the Figure.

Statistical analysis was performed using the χ² test. Two values were considered significantly different when P<.05.

**RESULTS**

At the termination of follow-up, we collected and analyzed the data. Nine patients developed local recurrence and neck lymph node metastasis, 6 developed local recurrence and lung metastasis, 5 developed bone metastasis, 5 developed brain metastasis, and 24 died. Histologic examination findings showed the carcinoma to be undifferentiated in 11 patients, poorly differentiated in 16, moderately differentiated in 12, and well differentiated in 12.

Positive immunostaining for carcinoma cells was present in 61% (31/51) and 57% (29/51) of the tumors for Bcl-2 and c-Myc protein, respectively. Tumor epithelial cell immunostaining for Bcl-2 was cytoplasmic and granular, and c-Myc protein was usually found in the nucleus and only rarely in cytoplasm. No certain correlation was established between expression of these proteins and histologic grades (staining scores, P= .14) (Table 1).

A reduced incidence of recurrence (death) and increased 5-year survival rates are significantly related to the presence of c-Myc+ staining (P=.02) or Bcl-2– staining (P=.02). Tumor recurrences were observed in 20 (65%) of 31 cases in the Bcl-2 staining group and 7 (35%) of 20 cases without expression (χ²=4.25; P=.06). Conversely, in the c-Myc+ group, 11 (38%) of 29 cases developed recurrent disease, and in the c-Myc– group, 16 (73%) of 22 cases had tumor recurrences (χ²=6.07; P=.03).
Expression of Bcl-2 and c-Myc also significantly correlates with patient outcome. The 5-year death rates were 61% (19/31) and 64% (14/22) for patients with Bcl-2+ and c-Myc− tumors, respectively, and 25% (5/20) and 34% (10/29) for those with Bcl-2− and c-Myc+ tumors, respectively (χ² = 6.42 and 4.27, respectively).

The probabilities of recurrence and death were higher in patients with Bcl-2+ tumors than in those with Bcl-2− tumors (odds ratio, 3.38 and 4.75, respectively). The probabilities of recurrence and death in patients with c-Myc+ tumors were lower than those in patients with c-Myc− tumors (odds ratio, 0.23 and 0.3, respectively).

Interval estimations are given in Table 2 and Table 3. Each of the 2 markers have some value in predicting patient outcome; in particular, when used simultaneously, the significance is greater.

The Kaplan-Meier curves showing disease-free and overall survival with respect to the expression of Bcl-2+/c-Myc−, Bcl-2+/c-Myc+, Bcl-2−/c-Myc+, and Bcl-2+/c-Myc+ are shown in survival function (Figure). In group 1.00 (Bcl-2+/c-Myc+), the survival rate dropped dramatically and frequently from the second year (100%) to the third year (70%) but remained stable from the third year to the fifth year. In group 2.00 (Bcl-2−/c-Myc−), the survival rate dropped obviously and frequently from the third year to the fourth. In group 3.00 (Bcl-2+/c-Myc−), the survival rate declined 20% in the third and fourth years. In group 4.00 (Bcl-2−/c-Myc−), the survival rate was 100% in the fifth year.

The significance of expression of the 2 biomarkers with respect to T stage and histologic grade was demonstrated using multiple analysis of variance. The homogeneity test for a single variable was carried out using the Levene test of equality of error variances (F_{histologic grade} = 0.39 and F_{T stage} = 1.35; P > .05 for both); it indicated that these data study coincided with the homogeneity test for variance.

Analysis for single-variable histologic grade or T stage was carried out using tests of between-subject effects (F_{histologic grade} = 1.8 and F_{T stage} = 0.381; P > .05 for both); it indicated that among different biomarker expression groups (Bcl-2+/c-Myc+, Bcl-2+/c-Myc−, Bcl-2−/c-Myc+, and Bcl-2−/c-Myc−), the histologic grade effect and the T stage were not significant.

The multivariate test result shows a Wilks Λ value of 1.05 and a Hotelling trace value of 1.05 (P > .05 for both); it indicated that when the analysis the body as a whole, between histologic grade and T stage, the different biomarker expression (Bcl-2+/c-Myc+, Bcl-2+/c-Myc−, Bcl-2−/c-Myc+, and Bcl-2−/c-Myc−) was not significant.

Concerning single-variable histologic grade or T stage, multiple comparisons were carried out among the different biomarker expression groups (group 1, Bcl-2+/c-Myc+; group 2, Bcl-2+/c-Myc−; group 3, Bcl-2−/c-Myc+; and group 4, Bcl-2−/c-Myc−). For histologic grade, the mean difference between group 1 and group 2 is 0.44, between group 1 and group 3 is 0.69, and between group 1 and group 4 is 0.94. The difference among different groups is not significant (P > .05). For T stage, the mean difference between group 1 and group 2 is 0.02, between group 1 and group 3 is −0.03, between group 1 and group 4 is 0.13. The difference among different groups is not significant (P > .05).

It has been suggested that the rate of tumor growth is determined by the balance between cell proliferation and cell death: only when the rate of proliferation exceeds that of death does tumor growth occur. Irradiation, there-

### Table 1. Relationship Between the Immunostaining Results and the Histologic Types

<table>
<thead>
<tr>
<th>Histologic Types</th>
<th>Patients, No.</th>
<th>Positive for Bcl-2</th>
<th>Negative for Bcl-2</th>
<th>Rate, %</th>
<th>Positive for c-Myc</th>
<th>Negative for c-Myc</th>
<th>Rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>42</td>
<td>6</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>50</td>
<td>6</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td>56</td>
<td>9</td>
<td>7</td>
<td>56</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>82</td>
<td>2</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>29</td>
<td>22</td>
<td>61</td>
<td>29</td>
<td>22</td>
<td>57</td>
</tr>
</tbody>
</table>

*Control slides consistently failed to demonstrate nonspecific binding. Tumor epithelial cell immunostaining for Bcl-2 protein was cytoplasmic and granular.

### Table 2. Interval Estimation for Patient Outcome for 51 Patients

<table>
<thead>
<tr>
<th>Marker Result</th>
<th>n</th>
<th>P Value (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 Recurrence</td>
<td>27</td>
<td>.53 (.39-.66)</td>
</tr>
<tr>
<td>Bcl-2 No recurrence</td>
<td>24</td>
<td>.47 (.34-.61)</td>
</tr>
<tr>
<td>c-Myc Recurrence</td>
<td>27</td>
<td>.53 (.39-.66)</td>
</tr>
<tr>
<td>c-Myc No recurrence</td>
<td>24</td>
<td>.47 (.34-.61)</td>
</tr>
<tr>
<td>Bcl-2 Death</td>
<td>24</td>
<td>.47 (.34-.61)</td>
</tr>
<tr>
<td>Bcl-2 Survival</td>
<td>27</td>
<td>.53 (.39-.66)</td>
</tr>
<tr>
<td>c-Myc Death</td>
<td>24</td>
<td>.47 (.34-.61)</td>
</tr>
<tr>
<td>c-Myc Survival</td>
<td>27</td>
<td>.53 (.39-.66)</td>
</tr>
</tbody>
</table>

### Table 3. Interval Estimation for Marker Staining for 51 Patients

<table>
<thead>
<tr>
<th>Marker Result</th>
<th>n</th>
<th>P Value (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 Positive</td>
<td>31</td>
<td>.61 (.47-.73)</td>
</tr>
<tr>
<td>Bcl-2 Negative</td>
<td>29</td>
<td>.39 (.27-.53)</td>
</tr>
<tr>
<td>c-Myc Positive</td>
<td>29</td>
<td>.57 (.43-.70)</td>
</tr>
<tr>
<td>c-Myc Negative</td>
<td>22</td>
<td>.43 (.31-.57)</td>
</tr>
</tbody>
</table>

### COMMENT

It has been suggested that the rate of tumor growth is determined by the balance between cell proliferation and cell death: only when the rate of proliferation exceeds that of death does tumor growth occur. Irradiation, there-
fore, may potentially act on responsive cancer cells by raising their rate of apoptosis, lowering their rate of proliferation, or both. It was found that the spontaneous level of apoptosis in nonirradiated murine tumor has been correlated with the tumor’s peak apoptosis response to irradiation. In this model, sensitivity of NPC to irradiation could be feasibly reduced by aberration in either the factors governing the regulation of apoptosis or the mechanisms associated with cell proliferation.

Patients with NPC are still facing low long-term survival rates. Eventually, most patients will die of the disease, which by then turns out to be radiation resistant. We report herein on Bcl-2 and c-Myc expression in a large cohort of patients with NPC, all of whom were treated and followed up in a uniform fashion.

This study included a homogeneous population of 51 patients with NPC stage squamous cell carcinoma treated by identical irradiation. We know that the tumor N stage before therapy had a prognostic impact, so the criterion of patients included in this study was absence of metastasis to determine whether expression of c-Myc and expression of Bcl-2 are independent prognostic indicators.

This study confirms that there is overexpression of Bcl-2 and c-Myc protein in NPC at the time of diagnosis. The prevalence of Bcl-2 and c-Myc positivity in our material was 73% (37/51) and 76% (39/51), respectively. The presence of Bcl-2 and c-Myc protein in NPC is statistically significantly related to prognosis: few patients with Bcl-2+ tumors survived for 5 years, with most developing local recurrence or distant metastases; on the other hand, patients with c-Myc+ tumors had a longer disease-free period. The authors collected some references and analyzed the information, intending to give a reasonable explanation for the results.

The bcl-2 gene seems to be of particular importance because it is known to be a key factor in increasing cell survival in several tumor types. Overexpression of the Bcl-2 product seems to prevent apoptotic events induced by irradiation. The present study suggests the role of Bcl-2 protein expression in the modulation of response to irradiation, since the presence of the protein and inhibition of the apoptotic pathway, which can extend the life span of tumor cells and decrease the overall therapeutic response to radiotherapy, might feasibly increase the chance of residual diseases after therapy. So the probability of tumor recurrence and patient death was greater.

Data published to date indicate that oncogene amplification is one of the most common genetic alterations found in human cancers. Examination of regional c-myc gene amplification within breast tumors showed that alteration of this gene can occur at an early in situ stage of tumor progression and often does not persist in late-stage nodal metastasis. The product of the c-myc oncogene is an important nuclear DNA-binding protein that seems to play critical roles in the regulation of cell growth and division. The c-Myc oncoprotein is activated to cause cell transformation by overexpression, resulting in intracellular accumulation. The observed relationship between an elevated level of c-Myc protein and a lower incidence of metastases to axillary lymph nodes for breast cancers without an altered c-myc gene may indicate that c-Myc protein also plays a significant role in the early stages of breast cancer development. In our study, we counted only the 64-kDa (likely active) c-Myc protein. Data presented in this study may support the assumption that alterations in the c-Myc protein level are involved in cancer progression, but in the early stage. So there is c-Myc overexpression at the time of tumor presentation, and this overexpression has no direct relation to the recurrence, which was a later biological behavior developed at an advanced stage of tumor. The recurrence rate in the c-Myc+ group was lower than that in the c-Myc− group. Conversely, a recent development in the biological behavior of c-Myc protein indicates that besides being a transcriptional factor important for cell growth and differentiation, in some circumstances c-Myc plays a crucial role in programmed cell death. It was found that irradiation-induced apoptosis is significantly increased when c-Myc is expressed. Therefore, the low recurrence rate observed for tumors with overexpression of c-Myc protein can also be the result of the proapoptotic activity of elevated c-Myc protein levels. We inferred that overexpression of c-Myc protein was enough to lead to apoptosis induced by irradiation.

On the other hand, some unknown genetic alterations accompanying c-myc gene amplification may overrule the proapoptotic action of c-Myc protein and lead to tumor progression. For example, a high concentration of Bcl-2 protein could protect cells from apoptosis induced by c-Myc protein. This could explain some situations presented in this study, which show there was also recurrence in some tumors with c-Myc protein overexpression. The precise mechanism responsible for apoptosis associated with overexpression of c-Myc protein is not clear.

This study has the advantage that the results can be analyzed in conjunction with extensive clinical follow-up data, so the result was reliable. The data showed that the 2 oncogenes had a close relation to NPC, as they were susceptible predictors of prognosis. An important and interesting finding was that the expression of Bcl-2 and c-Myc in pretreatment specimens was significantly correlated with the survival of radiation-treated patients with NPC; our data confirm this condition in patients with NPC for the first time, to our knowledge.

Overexpression of Bcl-2 protein could “protect” cancer cells from apoptosis induced by irradiation or could decrease the therapeutic response, so patients with Bcl-2+ NPC had significantly poorer survival rates; on the other hand, c-Myc protein is involved in cancer progression, but only in the early stage; the overexpression of c-Myc protein could “accelerate” apoptosis induced by irradiation, and since alterations in apoptosis rather than cell proliferation may be of vital importance in radiotherapy, patients with NPC had significantly longer disease-free survival in cases with c-Myc protein. Conventional radiotherapy was not effective enough for the Bcl-2+ tumor (or c-Myc– tumor) to be cured, so patients with this kind of tumor were inclined to experience recurrence and die. Expression of Bcl-2+ and c-Myc– might be related to residual disease after irradiation.
The 2 oncoproteins might be the important performer of cancer biological behavior; the manifestation of disease severity coming from their determination should be more objective and accurate. Clinically, expression of Bcl-2 and expression of c-Myc were important phenomena reflecting illness condition and predict recurrence (death). The biological functions and mechanisms of the 2 oncoproteins are different, and the analysis and multiple use of the resources can help the clinician in treatment. Determination of the 2 oncoproteins simultaneously would provide some useful reference data: the higher the expressions, the more severe the illness condition; the clinical significance is great.

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REFERENCE