Loss of cyclin D1 and p16 expression correlates with local recurrence in nasopharyngeal carcinoma following radiotherapy

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Received 24 September 2001; revised and accepted 11 February 2002

Background: The cyclin D1/p16/Rb pathway plays a critical role in tumorigenesis and each component of this pathway may be affected in various malignancies. The purpose of this study was to investigate the expression and prognostic significance of these proteins in nasopharyngeal carcinoma (NPC).

Patients and methods: Sixty-five patients undergoing radiotherapy for NPC were analyzed. The expression of cyclin D1, p16 and pRb was evaluated with immunohistochemical analysis of archived pretreatment tumor materials and expression of these proteins was correlated with clinicopathological parameters.

Results: Positive expression of cyclin D1 was observed in 43 of 65 NPCs (66%). p16 and pRb inactivation was identified in 42 of 65 (65%) and four of 65 (6%) tumors, respectively. All but seven tumors (58 of 65, 89%) contained at least one alternation in the cyclin D1/p16/Rb pathway. Loss of cyclin D1 as well as p16 was closely related to local recurrence after radiotherapy for NPC (P = 0.015 and 0.047). No association between pRb expression and clinicopathological outcome was apparent.

Conclusions: The study’s results suggest that the cyclin D1/p16/Rb pathway plays an important role in NPC tumorigenesis. We also find that cyclin D1 and p16 protein levels in NPC may be of use clinically as a predictor of local tumor control.

Key words: cyclin D1, local recurrence, nasopharyngeal carcinoma, p16

Introduction

Regulation of the G1–S transition of the cell cycle is tightly controlled by Rb pathway proteins, which include cyclin D1, p16 and Rb [1–3]. The retinoblastoma protein (pRb) inhibits the progression of cells into S phase and is regulated via pRb phosphorylation by the cyclin D1/cyclin-dependent kinase 4 (CDK4) complex. The cyclin D1/CDK4 complex phosphorylation activity is negatively regulated by the tumor suppressor gene p16. Each component of this cyclin D1/p16/Rb pathway can be affected in various malignancies [1, 2]. Abnormal expression of these cell-cycle regulators may affect not only the biological characteristics of the tumor but also its response to DNA-damaging therapies such as radiation.

Nasopharyngeal carcinoma (NPC) is derived from the nasopharyngeal epithelium, particularly from the fossa of Rosenmüller. There is a high prevalence of NPC in Southeast Asia, southern China, the Mediterranean basin and some North American indigenous populations [4]. NPC is generally considered to be unresectable owing to the typically awkward anatomical location of the tumor. Radiation therapy is therefore the primary treatment for all locally and regionally confined stages of NPC. Resistance to radiation therapy as defined by persistent disease after the course of radiotherapy is a poor prognostic factor in patients with NPC [5]. Of late, relationships between molecular genetic alterations in individual tumors and radiation sensitivity have been elucidated [6, 7]. Since NPC is characterized by remarkable infiltration of lymphocytes in the tumor tissue [8], molecular techniques directed at macroscopic tumor samples may be affected by normal tissue elements in the specimen [9]. Immunohistochemical analysis of the protein products of cyclin D1, p16 and Rb genes allows for their phenotypic localization at the cellular level in both frozen and archival tissues [10].

Positive expression of cyclin D1 and an absence of p16 are found in many different tumor types including breast carcinoma and squamous cell carcinoma of the head and neck [10, 11]. Abnormal expression of these proteins has been associated with a poor prognosis or recurrence in several reports.
A high frequency (64%) of p16 inactivation in NPC has been reported previously [15], whereas the expression of cyclin D1 in NPC has not. In this study, concurrent immunohistochemical analyses of cyclin D1, p16 and Rb gene products in primary NPC were employed to identify any correlation between the expression of these proteins and the tumor’s clinicopathological features and prognosis.

Patients and methods

Patients

There were 54 men and 11 women, with a mean age of 49 years (range 15–82 years). The UICC 1997 TNM staging system was used for clinical staging of the NPCs. All patients underwent local examination of the head and neck. Imaging studies that included bone scans, abdominal ultrasonography, computed tomography scan or magnetic resonance imaging were used to confirm the clinical staging. All patients received radiotherapy with equivalent doses of 68–75 Gy to the nasopharynx and 60–70 Gy to the neck. During follow-up over 6 years, imaging studies or biopsy results were used to verify distant or local recurrence of the tumor. Nine patients were lost to follow-up or died from other diseases. The total recurrence rate was 61% (34 of 56), including 30% (17 of 56) for local recurrence and 38% (21 of 56) for distant recurrence. The overall 5-year survival rate was 48% (27 of 56).

Immunohistochemistry

 Archived pretreatment tumor material was obtained from 65 patients with NPC. Five-micrometer sections were deparaffinized in xylene, rehydrated with graded alcohols, and then washed in phosphate-buffered saline (PBS). The sections were heated in 0.01 M citrate buffer (pH 4.0) in a microwave for 20 min. However, cyclin D1 and p16 assays were performed without a previous antigen retrieval method. Endogenous peroxidase was blocked with 3% hydrogen peroxide solution in methanol for 30 min. The primary antibodies were diluted in antibody diluent (Dako, Glostrup, Denmark) according to the following concentrations: anti-Rb mouse monoclonal antibody (Pharmingen, San Diego, CA, USA), 1:300; anti-p16 and anti-cyclin D1 mouse monoclonal antibody (Pharmingen), 1:100. The diluted antibodies were added and the slides were incubated overnight at 4°C. Slides were then washed in PBS and the reaction visualized with an UltraTech detection system (Immunotech, Marseilles, France). The slides were incubated with biotinylated secondary antibody at room temperature for 20 min followed by treatment with streptavidin-peroxidase reagent at room temperature for 20 min according to the manufacturer’s protocol. The peroxidase reaction was developed using 3-amino-9-ethyl carbazole solution (Immunotech). The slides were counterstained with hematoxylin and mounted. In addition, a corresponding section was treated with saline without the primary antibody as a control for non-specific staining. For the positive control, buccal carcinoma tissue with clear positive expression of cyclin D1 was used. With respect to pRb and p16, non-neoplastic cells served as the internal positive control for each biopsy.

Scoring for each marker was performed in a blinded fashion by two independent observers (C.-C. Huang and J.-S. Wang). Stains of pRb and p16 were interpreted using a previously described scoring method [15]. Only nuclear staining was evaluated; cytoplasmic reactivity, if present, was disregarded. A tumor was considered positive for pRb and p16 when there was distinct nuclear staining in all areas of the neoplasm (Figure 1).

A tumor was considered negative when there was no definite nuclear staining throughout the neoplasm relative to preserved nuclear reactivity in non-neoplastic cells of the same section.

Nuclear staining for cyclin D1 was non-uniform in all tumors, reflecting differences in expression during the cell cycle with a peak in G1 (Figure 2). Tumors were scored cyclin D1 negative if <5% of tumor cells exhibited nuclear staining and positive if >5% exhibited nuclear staining [16].

Statistical analysis

Associations between clinicopathological parameters and the cell-cycle-related proteins cyclin D1, p16 and pRb were evaluated using the t-test, chi-square test or Fisher’s exact test. The survival and recurrence rates were calculated using the Kaplan–Meier method and differences compared using the log-rank test. Results of analysis with a log-rank test P value of <0.05 were considered significant, and were included in a stepwise Cox
Using a proportional hazards model to assess the influence of these variables. All statistical tests were two-sided.

### Results

Positive staining for cyclin D1 was observed in cancer cell nuclei in 43 of the 65 cases (66%). The association between cyclin D1 levels and various clinicopathological features of the patients is summarized in Table 1. Patients with low levels of cyclin D1 exhibited a significantly higher rate of local recurrence than patients with high levels of cyclin D1 (Figure 3A; \( P = 0.015 \), log-rank test). No differences in cyclin D1 status were observed for other clinicopathological parameters or prognosis (Table 1).

Expression of p16 was positive in 23 tumors (35%) and negative in 42 tumors (65%). There was variable expression of p16 protein in the nuclei of benign cells, which included lymphoid cells, nasopharyngeal epithelium and fibroblasts. The association between p16 expression and various clinicopathological features is summarized in Table 1. Patients without expression of p16 exhibited a significantly higher rate of local recurrence than patients with detectable p16 (Figure 3B; \( P = 0.047 \), log-rank test). No differences in p16 status were observed for other clinicopathological parameters or prognosis (Table 1).

The variables evaluated by Cox multivariate analysis included sex, age, histology, tumor size, nodal stage, TNM staging, cyclin D1 and p16. The result showed that cyclin D1

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**Table 1. Correlation of cyclin D1 and p16 expression with clinicopathological status**

<table>
<thead>
<tr>
<th></th>
<th>Cyclin D1</th>
<th></th>
<th></th>
<th>p16</th>
<th></th>
<th></th>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>( P ) value</td>
<td>Positive</td>
<td>Negative</td>
<td>( P ) value</td>
</tr>
<tr>
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<td>22</td>
<td>–</td>
<td>23</td>
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<td>–</td>
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<tr>
<td>Male</td>
<td>33</td>
<td>21</td>
<td>0.082</td>
<td>20</td>
<td>34</td>
<td>0.733</td>
</tr>
<tr>
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<td>1</td>
<td>–</td>
<td>3</td>
<td>8</td>
<td>–</td>
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<tr>
<td>Age, years (mean ± SD)</td>
<td>48.4 ± 15.1</td>
<td>49.2 ± 12.0</td>
<td>0.810</td>
<td>51.9 ± 14.7</td>
<td>47.0 ± 13.4</td>
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<td>–</td>
<td>0.164</td>
<td>–</td>
<td>–</td>
<td>0.816</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>2</td>
<td>–</td>
<td>0</td>
<td>2</td>
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</tr>
<tr>
<td>II</td>
<td>24</td>
<td>10</td>
<td>–</td>
<td>12</td>
<td>22</td>
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</tr>
<tr>
<td>III</td>
<td>19</td>
<td>10</td>
<td>–</td>
<td>11</td>
<td>18</td>
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</tr>
<tr>
<td>Tumor size</td>
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<td>0.536</td>
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<td>0</td>
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<tr>
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</tr>
<tr>
<td>III</td>
<td>10</td>
<td>8</td>
<td>–</td>
<td>5</td>
<td>13</td>
<td>–</td>
</tr>
<tr>
<td>IV</td>
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<td>10</td>
<td>–</td>
<td>10</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>Local recurrence(^a)</td>
<td>7</td>
<td>10</td>
<td>0.015</td>
<td>2</td>
<td>15</td>
<td>0.047</td>
</tr>
<tr>
<td>Distant recurrence</td>
<td>13</td>
<td>8</td>
<td>0.175</td>
<td>6</td>
<td>15</td>
<td>0.581</td>
</tr>
<tr>
<td>5-year overall survival(^b) (months(^b))</td>
<td>17 (59.36)</td>
<td>10 (50.67)</td>
<td>0.538</td>
<td>9 (54.66)</td>
<td>18 (56.85)</td>
<td>0.580</td>
</tr>
</tbody>
</table>

\(^a\) The recurrence and survival rates were calculated by the Kaplan–Meier method and differences compared using the log-rank test.

\(^b\) Mean survival.

SD, Standard deviation; WHO, World Health Organization.
relative recurrence rate = 0.29, 95% confidence interval (CI) 0.11–0.76, $P = 0.012$) and p16 (relative recurrence rate = 0.22, 95% CI 0.05–0.96, $P = 0.044$) were significant independent factors associated with local recurrence.

Of the 65 patients in the series, 61 (94%) exhibited positive pRb staining. Staining in the remaining four patients (6%) was negative. pRb was expressed in a manner similar to that of p16 protein in adjacent benign tissue. No association between pRb expression and clinicopathological parameters or prognosis was apparent. If positive cyclin D1, negative p16 and pRb expression are regarded as abnormal expression, most NPC tissues (58 of 65, 89%) contained at least one abnormality in the expression of cyclin D1, p16 or pRb. When the combined expression of both p16 and cyclin D1 was addressed, local recurrence occurred in nine of 15 tumors with negative expression for both markers, which was the case in only eight of 41 tumors in which one or both markers were expressed ($P = 0.0002$, log-rank test).

**Discussion**

The high rate of positive cyclin D1 expression (66%) in NPC rivals that of head and neck squamous cell carcinomas in which a similar association has been seen, with amplification of cyclin D1 being reported in 30–64% of cases [12, 17, 18]. Various methods have been used to detect this amplification. High levels of cyclin D1 protein as detected by immunohistochemistry (IHC) occurred almost twice as frequently as cyclin D1 amplification [19]. This may be due to positive cyclin D1 expression resulting not only from amplification but also from other mechanisms of cyclin D1 gene expression deregulation [20]. In the present study, the higher positive rate for NPC tumors when cyclin D1 protein was analyzed with IHC is logical. In addition to this, 94% of the NPC specimens expressed pRb, whereas 65% lacked the p16 tumor suppressor protein. This may indicate that the Rb gene is rarely deleted or mutated; however, immunohistochemical stains are blind to the phosphorylation status of pRb, so identification of pRb in tissue sections does not exclude functional inactivation of this protein [21]. The mechanisms of p16 inactivation in NPC are usually deletion or methylation, but not point mutation, of p16 [22–24]. The present study extends these findings by demonstrating that the majority of NPCs had ceased to express p16 protein but still expressed pRb. If the expression of these proteins is looked at together, all but seven tumors contained at least one abnormality in the expression of cyclin D1, p16 or pRb. This observation confirms the fundamental role of the cyclin D1/p16/Rb pathway in NPC tumorigenesis.

NPC is a unique malignancy of the head and neck region that has a high sensitivity to both radiotherapy and chemotherapy. Despite advances in imaging technology and treatment techniques, several series have shown that local failure still occurs in 20–58% of patients receiving adequate treatment to the nasopharynx [25–27]. This highlights the importance of early prediction of local recurrence and the present study’s results suggest that IHC-identified reductions in cyclin D1 and p16 protein levels may be predictive of increased rates of local NPC recurrence after radiotherapy. Additional treatment may therefore be indicated to improve local control in such patients.

Studies have shown that T-stage, patient age and irradiation dose may be significant factors associated with local recurrence [28, 29]. Risk factors for local recurrence, which may reflect the intrinsic radioresistance and/or residual tumor
burden, are not necessarily the features that are predictive of distant metastases or overall survival. In this study, all patients were treated at a single institution in a highly uniform manner. The pretreatment clinicopathological status of positive and negative groups did not differ in a statistically significant manner. Both molecular markers (cyclin D1 and p16) were independently prognostic in patients at risk of local recurrence following primary radiotherapy. These results suggest that NPC with positive expression of cyclin D1 or p16 may have an improved response to radiotherapy (i.e. no occult tumor after radiotherapy).

The findings regarding cyclin D1 expression and radiation response are in keeping with recent studies. These studies have provided in vivo evidence demonstrating that low levels of cyclin D1 are associated with ipsilateral breast cancer and early-stage laryngeal cancer recurrence following radiation therapy [13, 30]. Pardo et al. [31] reported increased levels of apoptosis after irradiation of rat embryo cells that had been transfected with cyclin D1 and overexpressed the gene. Coco Martin et al. [6] reported that high levels of cyclin D1 altered the sensitivity of a breast tumor cell line towards ionizing radiation by modulating γ-radiation-induced G2–M transition. The tumor suppressor gene p16 is an important negative cell-cycle regulator whose functional loss may contribute significantly to malignant transformation and progression. Deletion of p16 has been associated with poor prognosis and relapse [14, 32]. Previous studies using tumor cell lines have shown a relationship between p16 expression and radiation sensitivity [7, 33], but other reports have failed to confirm these results [34, 35]. The present study provides in vivo evidence demonstrating that loss of p16 protein, seen in IHC, may mediate decreased response to radiotherapy in NPC. High levels of pretreatment p16 may be involved in blocking the cell cycle and increasing the proportion of cells in G1 phase. Radiosensitivity appears to increase in the G1 phase before the onset of DNA synthesis [36]. This may result in an increased response to radiation therapy and decreased local recurrence in NPC.

The results of the present study indicate that the cyclin D1/p16/Rb pathway plays an important role in NPC tumorigenesis. We also find that cyclin D1 and p16 protein expression may be useful clinically as a predictor of local tumor control in NPC. If the results of the present report can be confirmed in larger clinical and laboratory studies, molecular genetic alterations in individual tumors may eventually be able to guide therapeutic strategies.

Acknowledgements

The authors would like to express their appreciation to Dr Cheun-Chung Lui for data analysis. This study was supported by a research grant from the National Science Council of the Republic of China (NSC89-2314-B-182A-155), Taipei, Taiwan.

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