Cyclin D1 and p53 Overexpression Predicts Multiple Primary Malignant Neoplasms of the Hypopharynx and Esophagus

Takahide Kohmura, MD; Yasuhisa Hasegawa, MD; Tetsuya Ogawa, MD; Hidehiro Matsuura, MD; Masakatsu Takahashi, MD; Noriyuki Yanagita, MD; Tsutomu Nakashima, MD

**Background:** Multiple primary upper aerodigestive tract carcinomas can occur in up to 15% of patients. We have shown previously that half of the patients with multiple upper aerodigestive tract squamous cell carcinomas are initially seen with synchronous tumors. Most metachronous squamous cell carcinomas become manifest within 3 years.

**Objective:** To examine the expression of 2 proteins—cyclin D1 and p53—in an attempt to predict the occurrence of multiple primary malignant neoplasms (MPs).

**Materials and Methods:** Monoclonal antibodies to cyclin D1 (DCS-6 [dilution, 1:50], Novocastra Laboratories Ltd, Newcastle, England) and p53 (DO-7 [dilution, 1-100], Dako Corp, Carpinteria, Calif) proteins were used. Resection specimens from a total of 47 patients, 12 patients with MP and 35 patients with nonmultiple primary malignant neoplasms, were analyzed. Those in the nonmultiple primary malignant neoplasm group had longer than 3 years' follow-up to ascertain the absence of MP.

**Results:** Tumor overexpression of cyclin D1 was significantly associated with the development of MP (P < .01). Tumor overexpression of p53 was also frequent in patients with MP although statistical significance was not achieved. The combination of these 2 parameters was an even greater predictor of MP (P < .001).

**Conclusions:** Overexpression of cyclin D1 and p53 proteins was highly correlated with the development of MP. Additional studies are necessary to confirm this finding. Immunohistochemical evaluation of primary squamous cell carcinomas for cyclin D1 and p53 overexpression may become an important fact of surgical pathologic reporting for primary upper aerodigestive tract squamous cell carcinomas.

MATERIALS AND METHODS

RESECTION SPECIMENS

We retrospectively examined 104 patients with hypopharyngeal SCC who underwent initial treatment at the Department of Head and Neck Surgery, Aichi Cancer Center Hospital, Nagoya, Japan, between January 1, 1980, and May 31, 1996.6 Histological criteria for diagnosing multiple primary SCC, described by Warren and Gates,6 are as follows: (1) The neoplasms must be clearly malignant as determined by histological evaluation. (2) Each neoplasm must be geographically separate and distinct. The lesions should be separated by normal-appearing mucosa. If a second neoplasm is contiguous to the initial primary tumor or is separated by mucosa with intraepithelial neoplastic change, the 2 should be considered as confluent growths rather than multicentric carcinomas. (3) The possibility that the second neoplasm represents a metastasis should be excluded. The observation that the invasive carcinoma arises from an overlying epithelium that demonstrates a transition of carcinoma in situ to invasive carcinoma is helpful. When the separate foci have significant differences in histological features, the diagnosis of separate primary cancers is appropriate.

There were 12 patients with MP of the hypopharynx and esophagus; 7 were initially seen with synchronous tumors. In 4 of the 5 patients with metachronous tumors, the interval to the development of the second SCC was shorter than 3 years. Of the remaining 92 patients, we selected 35 patients who were followed up for longer than 3 years after having received primary treatment. This group (ie, those with nonmultiple primary malignant neoplasms [NMP]) was considered to be disease free of their primary SCC and was selected as the negative control for the MP group. All 47 patients had been operated on. Formalin-fixed, paraffin-embedded tissue was selected for study. The resection specimens from the MP group were compared with those of the NMP group. No statistically significant differences were noted in the stage of hypopharyngeal cancer between the MP (stage I, 0 cases; stage II, 1 case; stage III, 5 cases; and stage IV, 6 cases) and NMP (stage I, 4 cases; stage II, 9 cases; stage III, 14 cases; and stage IV, 8 cases) groups.

ANTIBODIES AND IMMUNOHISTOCHEMICAL STAINING

The primary antibodies used for staining were a mouse monoclonal antibody specifically for cyclin D1 protein (DCS-6 [dilution, 1:50]; Novocastra Laboratories Ltd, Newcastle, England) and a mouse monoclonal antibody specifically for p53 protein (DO-7 [dilution, 1:100]; Dako Corp, Carpinteria, Calif). Monoclonal antibody DO-7 detects nuclear accumulation of p53 that, in most cases (but not all), reflects expression of mutated p53.9,10 For immunohistochemical studies, a microwave retrieval treatment was applied to enhance the immunodetection of cyclin D1 and p53 proteins. The dewaxed and rehydrated sections were covered with citrate buffer (10 nmol/L [pH, 6]) and treated for 15 minutes at a 600-W setting of the microwave. The slides were then washed twice with a phosphate-buffered saline solution, subsequently stained with either DCS-6 or DO-7, and kept at 25°C overnight. The avidin-biotin complex procedure (ABC methods, OMNITAGS Kit; Lipshaw/Immunon, Pittsburgh, Pa) was used according to the manufacturer’s directions. Only nuclear staining for cyclin D1 and p53 proteins was regarded as positive. As shown in previous reports,7,11,12 in each specimen cyclin D1 and p53 expression were jointly scored, depending on the percentage of the nuclei staining (Table 1), by 2 of us (T.K. and T.O.) who were unaware of the clinical details. Four or more tumor fields were included in the examination. For statistical analysis, the specimens were categorized as positive or negative regardless of the degree of staining. We used the Fisher exact test to determine statistical significance, which was set at P<.05.

Table 1. Criteria of Immunohistochemical Staining

<table>
<thead>
<tr>
<th>Protein</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>- No nuclear immunoreaction</td>
</tr>
<tr>
<td></td>
<td>± Positive nuclei in up to 5% of the examined tissue</td>
</tr>
<tr>
<td></td>
<td>+ Positive nuclear staining ranging between 5% and 50%</td>
</tr>
<tr>
<td></td>
<td>++ Positive immunoreaction in &gt;50% of the nuclei</td>
</tr>
<tr>
<td>p53</td>
<td>- No nuclear immunoreaction</td>
</tr>
<tr>
<td></td>
<td>± Positive nuclei in up to 10% of the examined tissue</td>
</tr>
<tr>
<td></td>
<td>+ Positive nuclear staining ranging between 10% and 50%</td>
</tr>
<tr>
<td></td>
<td>++ Positive immunoreaction in &gt;50% of the nuclei</td>
</tr>
</tbody>
</table>

The occurrence of MP of the primary upper aerodigestive tract is well recognized, especially between the hypopharynx and esophagus. This has been traditionally explained by the “field cancerization effect” of generalized primary upper aerodigestive tract exposure to tobacco and alcohol. The p53 tumor suppressor gene encodes a product that blocks replication of damaged DNA by preventing entry into the S phase of the cell cycle.13 Cyclin D1 protein, the cell cycle control gene, is expressed during the G1 phase of the cell cycle6 and becomes associated with its catalytic partner cyclin-dependent kinase inhibitor 4 or 6.14 This association results in a serine or threonine kinase activity of cyclin D1 protein. The cyclin-dependent

either and negative of both) (Table 4 and Table 5). Combining both cyclin D1 and p53 parameters resulted in an even greater statistical significance for predicting MP (P<.001). All positive cases of both cyclin D1 and p53 proteins were included in the MP group. Examining these positive cases of both, no statistical association was noted between amount of staining and stage of the tumor (Table 3 and Table 4). This shows that there is a high possibility of development of MP in the positive cases of both cyclin D1 and p53 proteins. Also, more than half of the NMP cases stained negatively for both cyclin D1 and p53. Comparing Table 3 and Table 4 indicate that clear differences exist in the predictive power of both cyclin D1 and p53 overexpression vs cyclin D1 overexpression.

**COMMENT**
kinase inhibitor complex leads to a transit through the G1 phase, and cyclin D1 expression is rate limiting in this process. Overexpression of p53 and cyclin D1 have often been reported when prognosis and metastasis are considered. Although p53 mutation analysis has been studied in the differentiation between recurrence and MP,15 no studies have correlated MP with cyclin D1 and p53 analysis. In our study, we found significant differences in cyclin D1 overexpression for the MP and NMP groups. When we combined both cyclin D1 and p53 parameters, an even greater predictive power was achieved. Bradford et al10 reported that p53 immunostaining did not correlate with the p53 mutations as detected by single-strand conformation polymorphism analysis. Their report stated that immunohistochemistry identified p53 overexpression in some tumors with apparently normal single-strand conformation polymorphism patterns and, conversely, tumors with nonsense or frameshift mutations may not express any p53 detectable by immunohistochemistry. Actually, the relationship between p53 mutations and p53 overexpression may be more complex than previously thought.16 The hypothesis to explain these phenomena is that mutations are occurring downstream from p53, such as in WAF 1Cip 1.
a 21-kd protein that is transactivated by p53 and is an inhibitor of cyclin-dependent kinases. Interruption of the p53 pathway could interfere with a feedback loop that regulates expressions of wild-type p53. Overexpression of p53 might indicate an abnormality of the p53 pathway, an additional possible mutation. Therefore studying 2 or more parameters of G1-S phase may be meaningful. The predictive power for both cyclin D1 and p53 overexpression was more useful when compared with the predictive power of only cyclin D1 overexpression. These facts seem to prove the advantage of using 2 parameters. Nogueira et al have reported that samples with cyclin D1 and p53 abnormalities had a higher TNM stage and a more advanced tumor stage although they examined the amplification of cyclin D1 and the assay for the loss of heterozygosity of p53 protein. As more findings about the G1 pathway become available, we can find a more detailed relationship between cyclin D1 and p53 proteins.

We conclude that cyclin D1 and p53 parameters are useful in predicting MP. Further studies in other cases are needed to support our results. We also should perform a prospective study to confirm the occurrence of the secondary cancer by following up cases in which both cyclin D1 and p53 overexpression occurs.

Accepted for publication August 13, 1999.

Reprints: Takahide Kohmura, MD, Department of Otolaryngology, Nagoya University School of Medicine, 65 Tsu- rumai Showaku, Nagoya 466-8560, Japan.

REFERENCES