Expression of p21<sub>WAF1</sub> Predicts Outcome of Esophageal Cancer Patients Treated by Surgery Alone or by Combined Therapy Modalities

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ABSTRACT

The p21<sub>WAF1</sub> protein is an important regulator of the cell cycle. Its expression and prognostic significance were investigated immunohistochemically in samples of normal esophageal squamous epithelium (n = 10), severe squamous cell dysplasia (n = 20), carcinoma in situ (n = 14), permanent esophageal squamous cell carcinoma cell lines (n = 3), and invasive squamous cell carcinomas treated either by potentially curative resection (n = 172) or by combined modality therapy (radiochemotherapy ± surgery; n = 38). Whereas p21<sub>WAF1</sub> expression in the normal epithelium was restricted to a few cells adjacent to the basal cell layer, p21<sub>WAF1</sub> overexpression was frequently found in preneoplasias and invasive carcinomas. Expression of p21<sub>WAF1</sub> in invasive carcinomas was not correlated with tumor differentiation, pT category, or pN category. Among carcinomas treated by potential curative resection, univariate (P = 0.0025) and multivariate (P = 0.0081) survival analysis showed significant correlation of strong p21<sub>WAF1</sub> expression (≥50% p21<sub>WAF1</sub>-positive tumor cells) with poor overall survival. Univariate survival analysis (P = 0.0006) revealed the same prognostic influence in the group of patients treated by combined modality therapy. We conclude that overexpression of p21<sub>WAF1</sub> protein is a frequent event in preneoplasias and neoplasias of the esophagus. Immunohistochemical examination of p21<sub>WAF1</sub> expression may provide important prognostic information for decision-making in the treatment of patients with esophageal cancer.

INTRODUCTION

The prognosis of patients with esophageal cancer has only slightly improved during recent years. Even in resectable stages, the results of standard therapy modalities (surgery or radiotherapy) have been poor, with 5-year survival rates of about 20% (1).

The failure of standard therapy has motivated an increasing number of studies that have investigated combined treatment modalities including chemotherapy and/or radiotherapy and/or surgery in a variety of schedules. These studies have indicated that preoperative treatment with a combination of chemotherapy and irradiation produces a complete response in 20–40% of patients. However, only a few trials with combined modality treatment published thus far have shown an advantage over standard therapy in terms of overall survival and disease-free survival (2–4). A major problem in this context seems to be that lower rates of cancer-related deaths are counterbalanced by higher rates of treatment-associated mortality (5). For these reasons, it may be of great interest to find parameters that may help to identify those patients who might benefit from multimodal treatments and those who might not.

Factors influencing the result of radiochemotherapy in esophageal cancer have rarely been investigated thus far. Studies were largely focused on patient-related factors and therapy-related factors (e.g., tumor location and type of therapy: Refs. 6–8) and did not investigate the potential influence of tumor-related factors (e.g., proliferative activity and molecular parameters). In this regard, one of the most interesting tumor-associated factors may be p21<sub>WAF1</sub> (wild-type p53 activated fragment 1, also known as Cip-1, Cap20, Sdi-1, and Mda6) because of its function in the regulation of proliferation and programmed cell death (apoptosis). Initially, it has been shown that p21<sub>WAF1</sub> plays an essential role in the initiation of G1 arrest in cultured cells after exposure to DNA-damaging agents by either preventing phosphorylation of retinoblastoma protein (9–11) or by binding the proliferating cell nuclear antigen (12). In cell lines, induction of p21<sub>WAF1</sub> seemed to be dependent on functional p53 protein (9, 13–15). However, more recent in vitro data (16–18) and in vivo data (19, 20) suggest the presence of p53-independent pathways of p21<sub>WAF1</sub> induction. In addition to its cell cycle regulatory function, p21<sub>WAF1</sub> has been shown to protect cells from apoptosis, at least in certain in vitro models (21–23). Few data exist with regard to the potential role of p21<sub>WAF1</sub> in resistance to radiotherapy and/or chemotherapy. Thus, in colon cancer cells, loss of p21<sub>WAF1</sub> has been shown to correlate with increased sensitivity to irradiation (24, 25) and anticancer agents (26). In clinical samples from patients with acute myelogenous

Received 5/18/98; revised 8/10/98; accepted 8/11/98.

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leukemia, high levels of p21WAF1 were associated with resistance to chemotherapy (27).

In SCC of the esophagus, a recent immunohistochemical study of 22 invasive carcinomas revealed great intertumoral heterogeneity in p21WAF1 expression, showing the occurrence of tumors completely negative for p21WAF1 expression and tumors with overexpression of p21WAF1 (28). The influence of p21WAF1 expression on the outcome for esophageal cancer patients has not yet been determined. In the present study, we, therefore, investigated the expression of p21WAF1 and its prognostic significance in a series of 172 patients with SCC of the esophagus treated by standard therapy (surgery alone) and in a second series of 38 patients treated by combined treatment modalities (radiochemotherapy and — optionally — surgery). Additionally, p21WAF1 expression was investigated in samples of normal esophageal squamous epithelium, premalignant lesions (e.g., severe squamous dysplasias and carcinomas in situ), and continuous esophageal carcinoma cell lines to further determine the significance of p21WAF1 in esophageal SCC.

MATERIALS AND METHODS

Patients Treated by Surgery Alone. Tissue samples were collected retrospectively from 172 patients with SCC of the esophagus who had undergone potentially curative esophagectomy between January 1978 and December 1994. Potentially curative resection was defined as the absence of distant metastases, the removal of all of the gross tumor, and the histologically confirmed absence of tumor tissue at the surgical margins. No preoperative radio- or chemotherapy had been performed. Of the patients, 133 were male and 39 were female. The median age was 58 years (range, 24–82 years).

Pathological Review of the Surgical Samples. The esophagectomy specimens were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned, and stained with H&E. The pT and pN categories were determined according to the criteria proposed by the Union International Contre Cancer (29), and the grade of tumor differentiation was determined according to the criteria proposed by WHO (30). Tumor size was defined as the largest diameter of the tumor. Accordingly, 29 (16.9%) tumors were categorized as pT1, 33 (19.2%) as pT2, 105 (61.0%) as pT3, and 5 (2.9%) as pT4. Among all of the cases, 83 (48.3%) were in category pN0, and 89 (51.7%) were in category pN1. Of all of the tumors, 19 (11.1%) were graded as G1, 70 (40.7%) as G2, 52 (30.2%) as G3, and 31 (18.0%) as G4; 118 (68.6%) tumors had a maximum diameter of 5 cm or less, whereas 54 (31.4%) tumors were larger than 5 cm.

Moreover, severe squamous cell dysplasias synchronous with invasive carcinomas were found in 20 (11.6%) of the 172 cases, and carcinomas in situ were found in 14 (8.1%) of the 172 cases. Seventeen of 20 dysplasias and 8 of 14 carcinomas in situ were located adjacent to the invasive carcinomas, whereas 3 severe dysplasias and 6 carcinomas in situ were discontinuous from the invasive carcinomas, i.e., there was at least 1 cm of normal esophageal mucosa situated between the carcinoma and the dysplasia/carcinoma in situ.

Additionally, 10 samples of normal esophageal mucosa from the proximal resection margins of the esophagectomy specimens were selected for subsequent immunohistochemical investigations.

Patients with Multimodal Treatment. All patients formed part of a prospective multicentric treatment study. The results of this study, together with the criteria for patient selection and study design, have been described extensively elsewhere (31). For that reason, these features are described only briefly. All of the patients gave informed consent to participate in the study.

Patient Selection and Study Design. Patients with locally advanced esophageal cancer (i.e., category T3/T4 according to the Union International Contre Cancer classification (29) or obstructing tumors >5 cm in length in category T2), with or without regional lymph node metastases, were eligible.

Three courses of chemotherapy were administered within 9 weeks, followed by 4 weeks of radiotherapy with concomitant chemotherapy during the first 7 days. After the administration of chemotherapy and a cumulative dose of 40 Gy of radiotherapy, patients were either treated by an additional 20 Gy of radiotherapy (definitive radiochemotherapy) or by transthoracal esophageal resection. Postoperative treatment was not performed.

Preoperative Chemotherapy and Preoperative Radiochemotherapy. Chemotherapy consisted of FEP on days 1 to 3, every 3 weeks. Combined radiochemotherapy was started between day 22 and 28 of the last course of chemotherapy. The esophagus was irradiated using parallel-opposed anterior and posterior fields and photons from a 10- to 15-megavolt linear accelerator. A total dose of 40 Gy was given in daily fractions of 2 Gy, 5 times per week. During the first days of irradiation, the following chemotherapy was administered: cisplatin 50 mg/m² on days 2 and 8 and etoposide 100 mg/m² on days 4–6 (31).

Surgery. Resection of the esophagus and the proximal stomach was performed by a combined right thoracal and abdominal approach. Resection included excision of the paraesophageal, paracardial, left gastric, and celiac lymph nodes.

Criteria for Response to Chemotherapy. Response to chemotherapy was evaluated clinically after the third course and included evaluation of barium esophagogram, esophagoscopy, and computed tomography of the chest and abdomen. Response was categorized as complete response, partial response, no change, or progressive disease according to previously defined criteria (31). Data for tumor response after completion of radio- and chemotherapy were not available.

Pathological Review of Pretherapeutic Tumor Biopsies. Tumor biopsies that had been taken preoperatively were retrieved from the files of pathological institutes associated with the medical centers that took part in this study. Tumor biopsies from a total of 50 patients were collected. Of these 50 biopsies, 12 cases had to be excluded for the following reasons: (a) tumor type other than SCC (n = 8; 6 adenocarcinomas, 2 adenosquamous carcinomas); and (b) patients lost during follow-up (n = 4). This left 38 cases for further investigation.

Of these 38 patients, 31 were male and 7 were female. The
median age was 55 years (range, 42–70). Twenty-nine tumors had been clinically categorized as T3, and 9 tumors had been categorized as T4; 9 cases were in category N0, and 29 cases were in category N1.

Histological slides of the biopsy specimens were stained with H&E for the determination of tumor type and tumor grade (30). Two tumors were graded G1, 21 tumors were graded G2, and 15 tumors were graded G3.

**Cell Lines.** OSC-1 and OSC-2 are permanent cell lines derived from two poorly differentiated SCCs of the esophagus; they have been established and characterized in our laboratory (32). The esophageal SCC cell line Colo 680N (German Collection of Microorganisms and Cell Cultures; DSM ACC 182) was kindly provided by Dr. Martin Raida (Friedrich-Schiller-Universität, Jena, Germany). The cell lines were maintained in DMEM (Life Technologies, Karlsruhe, Germany). For the determination of p21WAF1 immunoreactivity, exponentially growing cells were washed twice in PBS and centrifuged (1500 rpm, 10 min). Pellets were fixed in 4% buffered formaldehyde and embedded in paraffin.

**p21WAF1 Immunohistochemistry.** p21WAF1 protein expression was investigated in the samples of normal esophageal mucosa, severe squamous cell dysplasia, carcinoma in situ, and invasive SCC as well as in the cell lines. After microwave pretreatment, slides were incubated overnight at 4°C with a monoclonal anti-p21WAF1 antibody (clone 6B6; PharMingen, Hamburg, Germany) at a dilution of 1:200. After a second incubation with a biotin-conjugated antimouse antibody, slides were incubated with an avidin-biotin-peroxidase reagent (Vector, Burlingame, CA). Reaction products were visualized by immersing slides in diaminobenzidine tetrachloride and finally counterstained with hemalaun. Positive staining of normal esophageal squamous epithelium provided an internal positive control for p21WAF1 staining.

The immunohistochemical expression of p21WAF1 was examined by means of light microscopy. The percentage of p21WAF1-positive tumor cells was determined semiquantitatively by assessing the whole tumor section, and each sample was assigned to one of the following categories: (a) 1 (0–4%); (b) 2 (5–24%); (c) 3 (25–49%); (d) 4 (50–74%); or (e) 5 (75–100%).

To define interobserver variability of this method, 20 samples of invasive SCCs were determined independently by two senior pathologists (M. Sa. and W. M.). This test showed full congruency in interpretation in 11 of the 20 cases. In nine cases, the two observers differed in one category. If 50% p21WAF1-positive tumor cells was used as the cutoff value to differentiate between tumors with low p21WAF1 expression and tumors with strong expression, concordance between the two pathologists was reached in 19 of the 20 cases, whereas discordance remained in only 1 case.

The remaining immunohistochemical samples were subsequently investigated by one pathologist (M. Sa.) without knowledge of the clinical outcome.

**Western Blot Analysis.** Four samples of esophageal SCCs (one with corresponding normal esophageal squamous epithelium) and exponentially grown OSC1, OSC2, and Colo 680N cells were snap-frozen in liquid nitrogen and stored at −80°C. The samples were homogenized in radioimmunoprecipitation assay buffer [150 mm NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 50 mm Tris (pH 8.0)] with protease inhibitors [leupeptine (1 μg/ml), aprotinin (1 μg/ml), pepstatine (0.5 μg/ml), and phenylmethylsulfonylfuoride (100 μg/ml)] and boiled for 5 min in Laemmlili-loading-buffer [62.5 mm Tris/HCl (pH 6.8), 2% SDS, 0.2 m DTT, 20% glycerol, and 0.001% bromphenol blue] at 95°C. Protein concentration was measured with the Bio-Rad DC Kit (Bio-Rad, Munich, Germany) according to the manufacturer’s instructions. One hundred μg of protein from each sample was subjected to electrophoresis in a SDS-polyacrylamide gel (12%) and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Inc., Bedford, MA). The membrane was incubated with the anti-p21WAF1 antibody (clone 6B6, PharMingen, Hamburg, Germany) at a dilution of 1:1000. Horseradish peroxidase-conjugated rabbit antimouse antibody was used as secondary antibody, and the bands were visualized using the Amersham ECL nonradioactive method according to the manufacturer’s instructions (Amersham Buchler, Braunschweig, Germany).

**Statistical Analysis.** Statistical analysis of the correlation between p21WAF1 expression and other parameters was performed by means of the two-sided Fisher’s exact test. Survival rates were calculated by the Kaplan-Meier method for analysis of censored data. The SDs in survival was analyzed by means of the log-rank test. The prognostic significance of parameters in multivariate analyses was determined by means of a forward Cox regression analysis; P < 0.05 were considered significant.

**RESULTS**

**Expression of p21WAF1 in Normal Esophageal Mucosa, in Severe Squamous Cell Dysplasias, and in Carcinomas in Situ.** In samples of normal esophageal squamous epithelium, nuclear p21WAF1 immunoreactivity was invariably found in two to three cell layers adjacent to the basal cells (Fig. 1a), whereas the other squamous epithelia were completely negative for p21WAF1. No p21WAF1 immunoreactivity was detectable in other normal tissues of the esophageal wall (e.g., smooth muscle cells or cells of esophageal mucous glands).

Nineteen (95.0%) of 20 severe squamous cell dysplasias and 12 (85.7%) of 14 carcinomas in situ showed expression of p21WAF1. In all of the severe dysplasias and carcinomas in situ that were positive for p21WAF1 expression, an upward shift of p21WAF1-positive cells toward superficial cell layers and an expansion of p21WAF1-negative basal cells were observed when compared with the normal esophageal squamous epithelium (Fig. 1b). The distribution of the severe dysplasias and carcinomas in situ with regard to the percentage of p21WAF1-positive tumor cells is detailed in Table 1.

**Expression of p21WAF1 in Invasive SCCs and in Esophageal SCC Cell Lines.** Of 172 surgically treated invasive carcinomas, 169 exhibited nuclear immunoreactivity for p21WAF1 (Fig. 1c), whereas 3 carcinomas were completely negative. In the 38 biopsies of SCCs that had been subsequently treated by radiochemotherapy, p21WAF1 expression was found in 35 cases; 3 cases were negative (Table 1).

All of the three esophageal SCC cell lines showed immu-
Fig. 1 Examples of p21^{WAF1} immunoreactivity (brown nuclear reaction product) in parabasal cell layers of normal esophageal squamous epithelium (A; ×260); in parabasal and superficial cell layers of a severe squamous cell dysplasia of the esophagus (B; ×320); in a moderately differentiated SCC of the esophagus (C; ×240); and in cells of the permanent esophageal carcinoma cell line OSC-1 (D; ×320).

Western Blot Analysis. Western blot analysis confirmed specificity of the p21^{WAF1} antibody used in this study, showing a single signal at M_r 21,000 in each sample that has been analyzed (Fig. 2). The three esophageal carcinoma cell lines displayed much stronger signals than the four samples of esophageal SCCs and the sample of normal esophageal squamous epithelium. OSC-1 cells and Colo 680N cells showed stronger signals than OSC-2 cells, corresponding to the data obtained by immunohistochemistry. The relatively weak signals of esophageal SCCs in Western analysis are most probably caused by a contamination of tumor-cell protein with protein from stromal
cells. This may also provide an explanation for an occasional inconsistency between signals obtained by Western blotting and the results of immunohistochemical analysis of esophageal SCC samples. Direct comparison of p21\textsuperscript{WAPl expression in normal esophageal squamous epithelium and corresponding carcinoma tissue was possible in one case, showing higher levels of p21\textsuperscript{WAPl} in the cancer tissue, both by immunohistochemistry and by Western analysis.

**Correlation between p21\textsuperscript{WAPl} Expression in Invasive Carcinomas and Response to Chemotherapy and Other Clinicopathological Parameters.** Upon division of the 38 patients who received 3 complete courses of FLEP chemotherapy into a group of responders (complete response or partial response) and a group of nonresponders (no change or progressive disease), tumors with low p21\textsuperscript{WAPl} expression (<50% positive tumor cells) were found more frequently in the first group of patients than in the latter one (81.3% versus 63.6%), whereas tumors with strong p21\textsuperscript{WAPl} expression (>50% positive tumor cells) were found to be less frequent in the group of responders than in the group of nonresponders (18.7% versus 36.4%). However, this difference did not attain statistical significance. No correlations were found when p21\textsuperscript{WAPl} expression in the 172 surgically treated carcinomas was analyzed in terms of pT category, pN category, and tumor differentiation (data not shown).

**p21\textsuperscript{WAPl} Expression and Survival of Surgically Treated Esophageal Cancer Patients.** The overall survival of all of the 172 patients has been followed regularly by the local tumor register up to May 1, 1997. Four patients were lost to follow-up, and 19 died of postoperative complications (within 30 days), leaving 149 patients for the survival analyses. At the end of the follow-up period, 33 (22.1%) of 149 patients were still alive. The follow-up time for all of the 149 patients ranged from 1–224 months after surgery (median, 19 months). The follow-up time for the 33 patients at risk ranged from 27–224 months after surgery (median, 62 months).

To determine the prognostic impact of p21\textsuperscript{WAPl} expression in a univariate survival analysis, patients were stratified according to the proportion of p21\textsuperscript{WAPl}-positive tumor cells as described in "Materials and Methods." In this analysis, we observed a continuous decrease of 2-year and 5-year survival rates from tumors with low percentages of p21\textsuperscript{WAPl}-positive cells to tumors with high percentages of p21\textsuperscript{WAPl}-positive cells (P = 0.0403). These differences in survival showed even higher significance under the log-rank test when the original five groups described above were combined into one group with less than 50% p21\textsuperscript{WAPl}-positive cells (2-year: 5-year survival, 46.8:27.1%) and one group with 50% or more p21\textsuperscript{WAPl}-positive cells (2-year:5-year survival, 12.0:8.0%; P = 0.0025; Table 2; Fig. 3A).

To determine whether p21\textsuperscript{WAPl} also has prognostic impact in a multivariate survival analysis, we performed a forward multivariate Cox regression analysis, including the parameters pT category, pN category, tumor grade, and p21\textsuperscript{WAPl} expression. The parameters that were not dichotomic were therefore dichotomized as follows: (a) pT category (pT1-pT2 versus pT3-pT4); (b) tumor grade (G1-G2 versus G3-G4); and (c) p21\textsuperscript{WAPl} expression (<50% positive cells versus ≥50% positive cells). In that analysis, p21\textsuperscript{WAPl} expression (P = 0.0081; relative risk, 1.80; 95% CI, 1.13–2.88), together with the parameters pN category (P = 0.0001; relative risk, 1.80; 95% CI,

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### Table 1

Expression of p21\textsuperscript{WAPl} in normal esophageal epithelium, severe squamous cell dysplasias, carcinomas in situ, invasive squamous cell carcinomas, and permanent cell lines of the esophagus

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>0-4%</th>
<th>5-24%</th>
<th>25-49%</th>
<th>50-74%</th>
<th>75-100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal esophageal epithelium</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>20</td>
<td>15.0</td>
<td>75.0</td>
<td>5.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>14</td>
<td>21.4</td>
<td>42.9</td>
<td>25.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Squamous cell carcinoma (resected specimens)</td>
<td>172</td>
<td>26.2</td>
<td>36.0</td>
<td>20.4</td>
<td>12.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Squamous cell carcinoma (pretherapeutic biopsies)</td>
<td>38</td>
<td>7.9</td>
<td>34.2</td>
<td>23.7</td>
<td>34.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Esophageal carcinoma cell line</td>
<td>3</td>
<td>0.0</td>
<td>0.0</td>
<td>33.3</td>
<td>66.7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

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### Table 2

Survival rates (%) by log-rank test of 149 surgically treated patients with SCC of the esophagus in relation to p21\textsuperscript{WAPl} expression

<table>
<thead>
<tr>
<th>p21\textsuperscript{WAPl}-positive tumor cells</th>
<th>n</th>
<th>2-yr (± SE)</th>
<th>5-yr (± SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4%</td>
<td>38</td>
<td>55.3 (±8.1)</td>
<td>36.1 (±7.9)</td>
<td>0.0403</td>
</tr>
<tr>
<td>5-24%</td>
<td>55</td>
<td>43.6 (±6.7)</td>
<td>22.9 (±5.9)</td>
<td></td>
</tr>
<tr>
<td>25-49%</td>
<td>31</td>
<td>41.9 (±8.9)</td>
<td>27.9 (±8.3)</td>
<td></td>
</tr>
<tr>
<td>50-74%</td>
<td>16</td>
<td>18.8 (±9.8)</td>
<td>6.3 (±6.1)</td>
<td></td>
</tr>
<tr>
<td>75-100%</td>
<td>9</td>
<td>22.2 (±13.9)</td>
<td>11.1 (±10.5)</td>
<td></td>
</tr>
<tr>
<td>0-4%</td>
<td>124</td>
<td>46.8 (±4.5)</td>
<td>27.1 (±2.2)</td>
<td>0.0025</td>
</tr>
<tr>
<td>50-100%</td>
<td>25</td>
<td>12.0 (±6.5)</td>
<td>8.0 (±5.4)</td>
<td></td>
</tr>
</tbody>
</table>
and Survival of Esophageal Cancer

Fig. 3 Overall survival in relation to expression of p21<sup>WAF1</sup> in the tumor tissue in 149 esophageal cancer patients treated by surgery alone (A) and in 17 esophageal cancer patients treated by radiochemotherapy and subsequent esophageal resection (B).

Table 3  Mean survival time by log-rank test of 38 patients with SCC of the esophagus treated with radiochemotherapy ± surgery in relation to p21<sup>WAF1</sup> expression

<table>
<thead>
<tr>
<th>p21&lt;sup&gt;WAF1&lt;/sup&gt;-positive tumor cells</th>
<th>Mean survival time (mo)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4%</td>
<td>3</td>
<td>14.0</td>
</tr>
<tr>
<td>5-24%</td>
<td>13</td>
<td>18.2</td>
</tr>
<tr>
<td>25-49%</td>
<td>9</td>
<td>10.2</td>
</tr>
<tr>
<td>50-74%</td>
<td>13</td>
<td>8.6</td>
</tr>
<tr>
<td>75-100%</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0-49%</td>
<td>25</td>
<td>18.9</td>
</tr>
<tr>
<td>50-100%</td>
<td>13</td>
<td>8.6</td>
</tr>
<tr>
<td>Definitive radiochemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-49%</td>
<td>10</td>
<td>10.5</td>
</tr>
<tr>
<td>50-100%</td>
<td>5</td>
<td>9.0</td>
</tr>
<tr>
<td>Radiochemotherapy and surgery&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-49%</td>
<td>12</td>
<td>23.8</td>
</tr>
<tr>
<td>50-100%</td>
<td>5</td>
<td>8.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Six patients received incomplete treatment.

DISCUSSION

The present study shows great intertumoral heterogeneity in the proportion of p21<sup>WAF1</sup>-expressing tumor cells among SCCs of the esophagus, whereas complete loss of p21<sup>WAF1</sup> expression is encountered infrequently. Our observation that a significant portion of invasive carcinomas displays p21<sup>WAF1</sup> overexpression as compared with the normal esophageal squamous epithelium is surprising, inasmuch as p21<sup>WAF1</sup> is thought to function as a tumor suppressor, and we had initially hypothesized that p21<sup>WAF1</sup> expression might be lost or down-regulated in SCC of the esophagus. However, the specificity of our results is underlined by the fact that we found similar patterns of p21<sup>WAF1</sup> expression in two independent series of invasive SCCs, one consisting of resection specimens and one consisting of biopsy samples. Moreover, we found a similar, although less pronounced, tendency for p21<sup>WAF1</sup> overexpression in a series of preneoplastic lesions of the esophagus. With regard to the normal esophageal epithelium, our results (expression restricted to a few layers of parabasal cells) perfectly correspond to the data published thus far (28, 33). Furthermore, the specificity of our immunohistochemical investigation was confirmed by additional Western blot analyses in esophageal cancer cell lines and in samples of normal esophageal squamous epithelium and esophageal SCCs.

Our results are further supported by two recently published immunohistochemical studies showing overexpression of p21<sup>WAF1</sup> in SCC of the skin (34) and in SCC of the head and neck (35). In the study of Yang et al. (28), who investigated 22 SCCs of the esophagus, loss of p21<sup>WAF1</sup> expression was found.

1.22–2.65), and pT category (P = 0.0166; relative risk, 1.64; 95% CI, 1.09–2.47), was shown to have independent prognostic impact for surgically treated esophageal cancer patients.

p21<sup>WAF1</sup> Expression and Survival of Esophageal Cancer Patients with Multimodal Treatment. The overall survival of all of the 38 patients had been followed up every 3 months for the first 3 years after the end of treatment and, subsequently, every 6 months. At the end of the follow-up period (April 15, 1997), 11 (28.9%) of 38 patients were still alive. The follow-up time for all of the 38 patients ranged from 3 to 64 months (median, 11 months). The follow-up time for the 11 patients at risk ranged from 8 to 64 months (median, 35 months).

When the prognostic impact of p21<sup>WAF1</sup> expression was analyzed in all of the 38 patients with multimodal treatment, a significant deterioration of survival with increasing percentage of p21<sup>WAF1</sup>-positive tumor cells was observed (P = 0.0050). The prognostic impact of p21<sup>WAF1</sup> was again of higher significance when the original five categories were combined into one category with less than 50% p21<sup>WAF1</sup>-positive cases and one category with 50% or more p21<sup>WAF1</sup>-positive cells (P = 0.0006). When the prognostic impact of p21<sup>WAF1</sup> was tested separately in the group with definitive radiochemotherapy and in the group with radiochemotherapy and subsequent esophageal resection, 6 of the 38 patients had to be excluded because they received incomplete treatment. In this analysis, p21<sup>WAF1</sup> retained its prognostic significance only marginally in the group with definitive radiochemotherapy (P = 0.0532), whereas in the group with radiochemotherapy and surgery, p21<sup>WAF1</sup> again showed highly significant influence on survival (P = 0.0002; Table 3; Fig. 3B).
in approximately equal frequency to \(p21^{\text{WAF1}}\) overexpression. Inconclusive results have been found in studies investigating the expression of \(p21^{\text{WAF1}}\) in adenocarcinomas of different locations. Thus, in adenocarcinomas of the lung (36) and in breast cancer (37), overexpression of \(p21^{\text{WAF1}}\) in tumor tissues was likewise found. In contrast, studies on ovarian cancer (37) and colorectal cancer (38) revealed a loss of \(p21^{\text{WAF1}}\) expression in these tumor types. Interestingly, Palazzo et al. (39) found a loss of \(p21^{\text{WAF1}}\) expression in adenocarcinomas of the endocervix and the endometrium, whereas in squamous cell dysplasias of the cervix uteri, an overexpression of \(p21^{\text{WAF1}}\) was determined. Taken together, the current data indicate that \(p21^{\text{WAF1}}\) is frequently overexpressed at the protein level in neoplasias and preneoplasias of squamous cell origin, whereas in adenocarcinomas, both overexpression and loss of expression occur.

The reasons for the apparent overexpression of \(p21^{\text{WAF1}}\) in some types of tumors as compared with their corresponding normal tissues are less straightforward. However, several considerations can be taken into account from the current knowledge of \(p21^{\text{WAF1}}\). Within normal cells, \(p21^{\text{WAF1}}\) exists predominantly in \(p21^{\text{WAF1}}\)/proliferating cell nuclear antigen (PCNA)/cyclin/CDK quaternary complexes that are able either to activate or to inhibit the CDK activity (40). \(p21^{\text{WAF1}}\) overexpression causes growth arrest only when it stoichiometrically exceeds the amount of cyclin-CDK complexes in the cell. Thus, \(p21^{\text{WAF1}}\) levels may represent a threshold that cyclin-CDK complexes have to overcome before the cell can enter into S phase. Overexpression of cyclins in tumor cells due to genetic aberrations (e.g., gene amplification) could result in a consecutive up-regulation of \(p21^{\text{WAF1}}\). In this context, it may be of interest that overexpression of cyclin D1 has been found in approximately one-third of SCCs of the esophagus (41).

On the other hand, it has been suggested that increased expression of \(p21^{\text{WAF1}}\) may be induced by the loss of other cyclin kinase inhibitors (e.g., p16\(^{\text{MTSI}}\), p15\(^{\text{MTS2}}\), and p27\(^{\text{KIP1}}\)) and reflects a feedback mechanism used by the tumor cells in an attempt to halt increased cell proliferation (20). More recently, a posttranscriptional mechanism for the accumulation of \(p21^{\text{WAF1}}\) has been proposed, inasmuch as, in contrast to the immunohistochemical analysis of \(p21^{\text{WAF1}}\) protein, in situ hybridization showed no increase of mRNA levels in head-and-neck cancer specimens (35). Finally, the detection of \(p21^{\text{WAF1}}\) expression does not necessarily indicate functional activity of the protein, inasmuch as it has been shown that \(p21^{\text{WAF1}}\) can be blocked by interaction with the HPV-16 E7 oncoprotein (42). In this connection, it has to be considered that overexpression of cellular \(p21^{\text{WAF1}}\) antagonists, e.g., Cdc25A (43), could also inhibit the function of \(p21^{\text{WAF1}}\).

Of even more clinical significance than the explanation for the reasons for \(p21^{\text{WAF1}}\) overexpression may be the potential prognostic influence of this phenomenon. In this regard, our investigation convincingly shows that the prognosis of surgically treated esophageal cancer patients deteriorates with increasing \(p21^{\text{WAF1}}\) expression in both univariate and multivariate survival analysis. Very little data exist thus far on the prognostic significance of \(p21^{\text{WAF1}}\) in other human malignancies. However, in good congruency with our data, a significantly shorter disease-free survival and overall survival has recently been shown for \(p21^{\text{WAF1}}\)-overexpressing tumors in a series of 42 SCCs of the head and neck (35). In contrast, \(p21^{\text{WAF1}}\) expression did not show any prognostic influence in breast cancer (44) and in thyroid cancer (45).

The negative prognostic influence of \(p21^{\text{WAF1}}\) overexpression in at least some types of tumors is surprising, inasmuch as \(p21^{\text{WAF1}}\) acts as a suppressor of proliferation and \(p21^{\text{WAF1}}\) expression should, therefore, be expected to positively influence the outcome of cancer patients. However, recent in vitro data indicate that in addition to its antiproliferative action \(p21^{\text{WAF1}}\) may counterbalance p53-induced apoptosis, thereby acting as a negative feedback mechanism in the apoptosis-inducing action of p53 (22, 23, 46). The suggestion, therefore, has been put forward that \(p21^{\text{WAF1}}\) overexpression (for whatever reason) may contribute to tumor progression by facilitating the growth of cells with sustained genomic instability and may contribute to treatment resistance by diminishing the cytotoxic effect of anticancer therapies that activate p53-induced apoptosis (46). This model would provide an explanation for: (a) the in vivo observation that, in acute myelogenous leukemia, \(p21^{\text{WAF1}}\) overexpression is associated with resistance to chemotherapy (27); and (b) a similar tendency found in our series of esophageal carcinomas.

Our observation that \(p21^{\text{WAF1}}\) may be of prognostic influence in esophageal cancer patients treated by combined therapy modalities is perhaps of greater clinical interest than its prognostic influence in patients treated by surgery alone. Thus, combined-modality treatment probably improves the chance of curing esophageal cancer patients, although the advantage over standard therapy (surgery and radiotherapy), in terms of overall survival and disease-free survival, has not been conclusively shown yet (2–4). Therefore, methods that may help to distinguish patients who will benefit from multimodal treatment from patients who will not would be of great clinical interest. Of course, our data are preliminary inasmuch as we have analyzed a relatively small number of patients who had been treated by two different treatment protocols. However, it remains an impressive finding of our study that no patient in the combined-modality treatment group whose tumor showed more than 50% \(p21^{\text{WAF1}}\)-positive cells survived more than 12 months after diagnosis.

If our data are confirmed by future prospective trials on a larger number of patients, a simple immunohistochemical investigation using endoscopically obtained tumor samples may provide information to the oncologist for the selection of patients either for intensive combined-therapy modality with curative intention or for palliative therapy.

**ACKNOWLEDGMENTS**

We thank S. Schneeloch and C. Golmina for perfect technical assistance.

**REFERENCES**

Expression of p21WAF1 predicts outcome of esophageal cancer patients treated by surgery alone or by combined therapy modalities.


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