The Expression of p53, p21 and TGF β1 in Gastric Carcinoma

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Summary: The aim of the present study was to examine the significance of the p21 expression in gastric cancer. We examined the expression of p53, p21, TGF β1 and PCNA in 75 cases of gastric cancer using immunohistochemical examinations and the expression of p21 RNA by in situ hybridization (ISH). The combination of p53 and p21 expressions was related to depth of invasion, lymph node metastasis, and stage grouping. The survival curves of the p53 negative-Group and the p21-positive Group were significantly higher than those of the p53-positive and the p21-negative Group, the p53-and-p21-both-positive Group, and the p53-and-p21-both-negative Group (each p<0.01). The average PCNA Labelling Index (LI) of the p53-negative-and-p21-positive Group was significantly lower than that of either the p53-positive-and-p21-negative Group or the p53-and-p21-both-positive Group or the p53-and-p21-both-negative Group (p<0.01, p<0.05, p<0.05, respectively). All of the p53-and-p21-both-positive cases were TGF β1 positive, and the rate of the TGF β1 positive cases in the p53-and-p21-both-positive Group was significantly higher than that of the p53-positive-and-p21-negative Group, and than the rate in the p53-and-p21-both-negative Group (each p<0.01). The survival curves of the cases with expression of p21 RNA were higher than that of cases without p21 RNA (p<0.05). Many of the p53-positive-and-p21-negative cases were advanced cancer with very poor prognosis, but many of the p53-negative-and-p21-positive cases were early cancer with good prognosis. These results suggest that p21 suppressed synthesis of DNA via PCNA, and TGF β1 is a regulation factor for the expression of p21, and that the combination of p53 and p21 expression is concluded to be a useful prognostic marker of gastric carcinoma.

Key words combination of p53 and p21 expression, TGF β1, PCNA, gastric carcinoma, p21 RNA

INTRODUCTION

Following DNA damage, p53 protein levels rise dramatically, and the entry into S is delayed until the genomic lesions are fully repaired. When the p53 function is lost, cells enter S without appropriate DNA repair, leading to fixation and propagation of genetic alterations. The cell cycle from the G1 phase to the S phase regulators such as cyclins, cyclin-dependent kinase (Cdks), PCNA and their inhibitors control the growth of the cells. p21 encodes a protein of Mw 21,000, which is a potent inhibitor of G1 Cdks whose expression is induced by p53 [1-4]. p53 overexpression promotes the transcription of p21, the product of which causes growth arrest through inhibition of Cdks, which are required for G1 to S transition [3]. Moreover, p21 controls DNA replication by interaction with PCNA [5], p21 is induced by DNA damaging agents that trigger G1 arrest or apoptosis in cells with wild type p53 but not in tumor cells harboring a deletion or mutation in the p53 gene [1,6]. Recently, the p53-independent p21 expression has been reported. Akagi et al. [7] examined the effect of TGF β on the expression of p21, G1 cyclins and Cdks using human gastric cancer cell lines, and con-
eluded that TGFβ was an inducer of p21.

The aim of the present study was to examine the significance of the p21 expression in gastric cancer. We examined immunohistochemically p21, p53 and TGFβ1 in gastric cancer tissue, and performed a detailed study of the patterns of the p21 RNA expression in the cancer regions using in situ hybridization (ISH).

MATERIALS AND METHODS

Tissues

Samples of gastric cancer were taken from the resected stomach of 75 patients who had undergone gastrectomy for gastric carcinoma, between 1990 and 1995. All patients were diagnosed histologically according to the General rules for Gastric Cancer Study in Surgery and Pathology of the Japanese Gastric Cancer Society [8]. The number at Stage I, Stage II, Stage III, and Stage IV, was 18, 6, 29, and 22, respectively.

Samples were obtained from the central zone of the cancer lesion and immediately flash froze this in liquid nitrogen. We made frozen sections of each sample, fixed in fresh 4% paraformaldehyde and stored at −80°C until required for immunohistochemistry and ISH.

Molecular probes

The p21 probe, a 2121-bp subcloned into the transcription vector pBluescript SK(+) (Stratagene, La Jolla, CA) was a kind gift from Dr. Asao Noda. The insert was excised using Bam H1 restriction enzyme and verified for size by agarose gel electrophoresis. After linearization of the plasmid, digoxigenin-labelled antisense and sense RNA probes were synthesized by in vitro transcription using T7 and T3 RNA polymerase (Boehringer Mannheim Corp, Germany) and UTP linked via aspacer arm to the steroid hapten digoxigenin according to the manufacturer’s directions (DIG RNA Labeling Kit, Boehringer Mannheim Corp, Germany).

In situ hybridization procedure

In each experiment, semiserial sections of the 24 tissues were hybridized with the antisense and sense p21 probes. The sections were soaked for 10 min at room temperature in 0.25% acetic anhydride in 0.1 M triethanolamine HCl. The sections were then hybridized overnight at 50°C in a mixture containing 50% deionized formamide, 10% dextran sulfate, 2×SSC, 20 μg/ml RNA, and then the digoxigenin-labeled antisense or sense p21 RNA probe (0.2 μg/ml) was diluted 10 times with hybridization solution. After a final strigency wash at 50°C in 0.2 SSC for 20 min, hybridization was examined immunohistochemically using components of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim Corp, Germany) where a positive signal resulted in a deep blue-brown staining. The tissues were counter-stained with methylgreen and overslipped for evaluation using light microscopy. Sections of the tissues that were hybridized with the sense p21 probes were used for the negative control with each staining.

Immunohistochemistry

Seventy-five sections were stained using the avidin-biotin-peroxidase technique (ABC method). In brief, after frozen sections were washed with PBS, sections were fixed in cold acetone for 10 min, washed with PBS again, and then immersed in methanol containing 0.3% H2O2 for 30 min to block endogenous peroxidase activity. The sections were then incubated with anti-p53 antibody (diluted 1:500 DAKO, Santa Barbara, CA, USA), anti-p21 antibody (diluted 1:500 Novocstra Laboratories, Newcastle, UK), anti-TGFβ1 antibody (diluted 1:500 Antigenix America, NY, USA) and anti-PCNA antibody (diluted 1:500 DAKO) for at least 12 hrs at 4°C followed by incubation with biotinylated rabbit anti-mouse serum for 30 min and incubation with streptavidin-peroxidase complex for 30 min. Staining was developed by incubating the sections in 3-amino-9-ethylcarbazole (AEC) for 5 min. The sections were then counterstained in hematoxylin, and mounted. Non-immuno serum was used for the negative control with each staining. The intensity of the immunohistochemical reaction was evaluated in at least ten random microscopic high power fields at 400× magnification. The absence of positive cells was scored as −, and more than 10% positive cells was scored as +. A total of 1000 cells was counted for each section, and the average percentage of stained cells (PCNA Labelling Index (LI)) was calculated.

Statistical analysis

The χ2 test was used for comparison of group frequencies as appropriate. p<0.05 by the two-tailed
test was considered statistically significant. Survival curves were computed using the Kaplan-Meier method and differences between survival curves were compared using the Cox-Mantel test. Differences were considered significant at the 5% level.

RESULTS

Immunohistochemical staining

Positive stainings of the p53 and p21 proteins were recognized in the nucleus of the cancer cells (Fig 1A, B). The number of tumors in each group that expressed the p53 and p21 proteins are shown in Table 1. The rate of lymph node metastasis in the p53(−) p21(+) Group was significantly lower than that in either the p53(+) p21(+) or the p53(−) p21(+) Group (each p<0.01). The rate of T1 cases in the p53(−) p21(+) Group was significantly higher than that in the p53(+) p21(+) or the p53(−) p21(+) Group (p<0.01, p<0.05, respectively). The rate of the T3 cases in the p53(−) p21(+) Group was significantly lower than that in either the p53(+) p21(+) or the p53(−) p21(+) Group (p<0.01, p<0.05, respectively). The rate of Stage I cases in the p53(−) p21(+) Group was significantly higher than that in the p53(+) p21(+) or the p53(−) p21(+) Group (each p<0.001). The rate of Stage III cases in the p53(−) p21(+) Group was significantly lower than that in either the p53(+) p21(−) or the p53(−)

Fig. 1A, B. Immunohistochemical analysis of p53 and p21 in gastric carcinoma A p53, B p21 ×100. Positive immunostaining was observed mainly in the nuclei.

TABLE 1.

<table>
<thead>
<tr>
<th></th>
<th>p53(−) p21(−)</th>
<th>p53(−) p21(+)</th>
<th>p53(+) p21(+)</th>
<th>p53(−) p21(+)</th>
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<tr>
<td></td>
<td>17 cases (17.3%)</td>
<td>11 cases (14.7%)</td>
<td>5 cases (6.7%)</td>
<td>42 cases (56.0%)</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (7.1%)</td>
</tr>
<tr>
<td>P</td>
<td>1 (5.9%)</td>
<td>0</td>
<td>0</td>
<td>6 (14.3%)</td>
</tr>
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<td>N</td>
<td>14 (82.4%)</td>
<td>2 (18.2%)</td>
<td>4 (80.0%)</td>
<td>32 (76.2%)</td>
</tr>
<tr>
<td>T1</td>
<td>6 (54.3%)</td>
<td>0</td>
<td>8 (19.0%)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>1 (5.9%)</td>
<td>4 (36.4%)</td>
<td>2 (40.0%)</td>
<td>6 (14.3%)</td>
</tr>
<tr>
<td>T3</td>
<td>14 (82.4%)</td>
<td>1 (9.1%)</td>
<td>3 (60.0%)</td>
<td>22 (52.4%)</td>
</tr>
<tr>
<td>T4</td>
<td>2 (11.8%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>St.I</td>
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<tr>
<td>St.II</td>
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<td>1 (20.0%)</td>
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<tr>
<td>St.III</td>
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<td>1 (9.1%)</td>
<td>4 (80.0%)</td>
<td>15 (35.7%)</td>
</tr>
<tr>
<td>St.IV</td>
<td>6 (35.3%)</td>
<td>0</td>
<td>0</td>
<td>16 (38.1%)</td>
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a,b,c,d,e,p<0.01 compared with p53(−) p21(−); a,b,c,d,f,i,k,p<0.05 compared with p53(−) p21(+); c,e,p<0.001 compared with p53(−) p21(+)
p21(+) Group (each p<0.05). The survival curves of the p53(−) p21(+) Group were significantly higher than those of the p53(+) p21(−), p53(+) p21(+), and the p53(−) p21(−) group (each p<0.01) (Fig. 2). The survival curves of the p53(+) p21(−) Group were significantly lower than those of the p53(−) p21(−) Group (p<0.01) (Fig. 2). The survival curves of Stage III cases in the p53(−) p21(−) Group were significantly higher than those in the p53(+) p21(−) or the p53(+) p21(+) Group (p<0.01, p<0.05, respectively) (Table 2).

The PCNA LI of the p53(−) p21(+) Group was significantly higher than either that of the p53(+) p21(−), p53(+) p21(+), and the p53(−) p21(−) Group (p<0.01, p<0.05, p<0.05, respectively) (Table 2).

All the p53(+) p21(+) cases were TGF β1(+), and the rate of TGF β1(+) of the p53(+) p21(+) Group was significantly higher than that of the p53(+) p21(−) and the p53(−) p21(−) Group (each p<0.01) (Table 2).

In situ hybridization

Positive staining p21 RNA was recognized in the cytoplasm of cancer cells using the digoxigenin labeled antisense p21 RNA probe. On the other hand, positive staining of p21 RNA was not recognized using the sense p21 RNA probe (Fig 4A, B). The expression of the p21 RNA was recognized in 17 cases (70.8%); in 8 of the 10 Stage I cases (80%), in 2 of the 2 Stage II cases (100%), in 7 of the 9 Stage III cases (77.8%), and in 0 of the 3 Stage IV cases (0%) (Table 3). The 5-year-survival rate of cases with expression of p21 RNA was 82.4%, and that of cases without expression of p21 RNA was 35.7%. The survival curves of cases with an expression of p21 RNA were higher than those of cases without expression of p21 RNA (p<0.05) (Fig. 5). The expression of p21 RNA was seen in 10 of the p21-protein-positive cases (100%), and in 7 of the p21-protein-negative cases (50%) (Table 3). The p53 protein was positive in 4 of the 17 cases with p21 RNA expression (23.5%), and in 2 of the 7 cases...
without p21 RNA expression (28.6%). The TGF β1 protein was positive in 11 of the 17 cases with a p21 RNA expression (64.7%), and in 1 of the 7 cases without p21 RNA expression (14.3%). There was no difference in the rate of the p53-protein-positive cases among those with p21 RNA expression and the rate in cases without p21 RNA expression, but the rate of TGF β1-positive cases in cases with p21 RNA expression was significantly higher than that of cases without p21 RNA (p<0.05).

**TABLE 3.**

<table>
<thead>
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<th>In situ hybridization</th>
<th>p21 RNA expression (+)</th>
<th>p21 RNA expression (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I (n=10)</td>
<td>8 (80.0%)</td>
<td>2 (20.0%)</td>
</tr>
<tr>
<td>Stage II (n=2)</td>
<td>2 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Stage III (n=9)</td>
<td>7 (77.8%)</td>
<td>2 (22.2%)</td>
</tr>
<tr>
<td>Stage IV (n=3)</td>
<td>0</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>p21 protein (+) (n=10)^a</td>
<td>10 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>p53 protein (+) (n=6)</td>
<td>4 (66.7%)</td>
<td>2 (33.3%)</td>
</tr>
<tr>
<td>TGF β1 protein (+) (n=12)^b</td>
<td>11 (91.7%)</td>
<td>1 (8.3%)</td>
</tr>
</tbody>
</table>

_a) p21 protein positive rate of cases with expression of p21 RNA was significantly higher than that of cases without expression of p21 RNA (p<0.05).

_b) TGF β1 protein positive rate with expression of p21 RNA tended to be higher than that of cases without expression of p21 RNA.

*Fig. 4A, B.* In situ hybridization for p21 RNA. A) p21 RNA positive staining was recognized in the cytoplasm of the cancer cells using the digoxigenin-labelled antisense p21 RNA probe. B) p21 RNA positive staining was not recognized using the sense p21 RNA probe. ×100
expression tended to be higher than in cases without p21 RNA expression (Table 3).

**DISCUSSION**

The p21 gene which is induced by p53 has been mapped to the p arm of chromosome 6 and is about 15kb in size [4], and the binding site of p53 protein exists upstream from the promotor site [1]. Cyclin-dependent kinase inhibitor p21 is recognized as a negative regulator of cell cycle progression from the G 1 phase to the S phase, and it possibly mediates cell differentiation and apoptosis. Xiong et al. [3] have reported that overexpression of p21 arrested the cell division.

Complexes are formed by G1 cyclins (C type, D type and E type cyclin) and Cdkks phosphatylate retinoblastoma (Rb) protein, then activate E2F which is a transcription factor, and then DNA replication enzymes such as DNA polymerase a are induced which promote the cell cycle from the G1 phase to S phase [9-11].

Increasing amounts of p21 protein resulted in an accumulation of cyclins (A type, D type and E type cyclin), CDK2 kinases, and p21 ternary complexes, with a corresponding inhibition in Rb phosphorylation and inactivation in E2F, which induced G1 arrest or apoptosis [1,6]. Waga et al. [5] have reported that p21 protein controlled DNA replication through interaction with PCNA. In the present immunohistochemical study, many in the p53(+) p21(-) Group were advanced cancer cases, with a poor prognosis. However, many in the p53(-) p21(+) Group were early cancer cases, with a good prognosis. Moreover, the PCNA Labelling Index of the p53(-) p21(+) Group was significantly lower than that of the other groups. In this study on the expression of p21 RNA using ISH, the rate of Stage I or II cases with expression of p21 RNA was high, and the 5-year survival rate of cases with expression of p21 RNA was higher than that of cases without expression of p21 RNA. These results suggested that the regulation system of the cell cycle did not work in the p53(+) p21(-) cases, and that p21 suppressed the synthesis of the DNA via PCNA. TGF βs are a group of multifunctional growth factors that inhibit cell cycle progression in many cell types [12-15]. The TGF β-induced cell cycle arrest has been partially attributed to the regulatory effects of TGF β on both the levels and activities of the G1 cyclins and their cyclin-dependent kinase partners [16-18]. The ability of TGF β to inhibit the activity of these kinase complexes derives in part from its regulatory effects on the cyclin-dependent kinase inhibitors p21, p27 kip1, and p15 [19,20]. After treatment of cells with TGF β, these three inhibitors bound to and blocked the activities of the specific cyclin-cyclin-dependent kinase complexes to cause cell cycle arrest [19,20]. In the case of p21, TGF β treatment leads to an increase in p21 mRNA [21-23]. This increase in p21 mRNA was partly due to transcriptional activation in the p21 promotor [21]. Datto et al. [24] defined a 10-base pair sequence that is required for the activation of the p21 promotor by TGF β. Akagi et al. [7] examined the effect of TGF β1 on the expression of p21, G1 cyclins and cdkks, using human gastric cancer cell lines. They reported that TGF β1 induced p21 expression and subsequently suppressed cdk2 kinase activity, followed by a reduction in phosphorylation of the product of the Rb tumor suppressor gene in TMK-1 cells, and that TGF β1 did not affect the level of p53 mRNA or TMK-1 cells, which contained mutated p53 genes [7]. In our study, all the p53(+) p21(+) cases were TGF β1(+), and the rate of TGF β1(+) in the p53(+) p21(+) Group was significantly higher than either that of the p53(+) p21(−) or the p53(−) p21(−) Group. There was no difference in the p53- protein-positive rate between those with a p21 RNA expression and those without a p21 RNA expression, but the rate of TGF β1 in the cases with a p21 RNA expression was more than in cases without a p21 RNA expression (p<0.1). These results suggested that TGF β1 was important for the regulation of p21 in a p53-independent pathway. However, the prognosis of the p53(+) p21(+) group was not so good, because other regulators of the cell cycle
failed to regulate the cell cycle progression. These results suggested that p21 suppressed synthesis of DNA via PCNA, and that TGF-β1 was one of the regulating factors for the expression of p21. A combination of p53 and p21 was concluded to be a good prognostic marker of gastric carcinoma.

REFERENCES