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**Ha-ras ONCOGENE PRODUCT IN HUMAN GASTRIC CARCINOMA: CORRELATION WITH INVASIVENESS, METASTASIS OR PROGNOSIS**

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C-Ha-ras oncogene product in human gastric carcinomas was examined by Western blotting and immunohistochemistry using anti-Ha-ras p21 antibody. In Western blotting, high levels of c-Ha-ras p21s were found in gastric carcinomas. Immunohistochemically, C-Ha-ras p21 was detected in 3 (11.1%) of 27 early carcinomas and in 63 (43.8%) of 144 advanced carcinomas. In advanced carcinomas, C-Ha-ras p21-immunoreactivity was correlated with the depth of tumor invasion and was stronger in metastatic tumors than in primary tumors. Patients with C-Ha-ras p21-positive carcinomas had a significantly worse prognosis than those with p21-negative carcinomas.

Key words: C-Ha-ras p21 — Gastric carcinoma — Prognosis — Immunohistochemistry

The ras gene family is comprised of Ha-, Ki- and N-ras genes, which code GTP-binding proteins of 21,000 daltons (p21).1) Ras p21 is known to be associated with the inner surface of the plasma membrane and to exhibit GTPase activity.2) Moreover, ras p21 shares limited sequence homology with G proteins which mediate stimulation and inhibition of adenyl cyclase following the attachment of polypeptide hormones to specific surface receptors.3) Point mutation in c-ras genes, which frequently occurs in codons 12 and 61, and the enhanced expression of normal c-ras gene have been found in various human solid tumors involving the colon, urinary bladder and lung.4) More recently, an immunohistochemical study has demonstrated that c-ras p21 expression in prostatic cancer correlates with histologic tumor grade and that ras p21 serves as a tissue tumor marker for determining the prognosis of patients with prostatic cancer.5) Moreover, the Ha-ras oncogene has been shown to confer experimental metastatic ability on nonmetastatic NIH 3T3 cells.6) In the present study using Western blotting and immunohistochemical techniques, we examined the presence of c-Ha-ras p21 in human gastric carcinoma in an attempt to determine the correlations between c-Ha-ras p21-immunoreactivity and the depth of tumor invasion, metastasis and prognosis.

A total of 171 gastric carcinomas including 27 cases of early carcinoma and 144 cases of advanced carcinoma were used. They were surgically resected at Hiroshima University Hospital and Kure Mutual Aid Hospital during the 9-year period from 1976 to 1984. All of these carcinomas were fixed in 10% neutral formalin and embedded in paraffin. Representative blocks were selected and serial sections were prepared. In some cases of advanced carcinoma, tumor tissue and nonneoplastic gastric mucosa were removed immediately after resection, frozen in liquid nitrogen and stored at -80° for Western blotting. Definition of stage grouping and histological classification of gastric cancer were made according to the criteria of the Japanese Research Society for Gastric Cancer.7)

For the detection of C-Ha-ras p21 within the tumors, a modification of the immunoglobulin enzyme bridge technique (avidin-biotin complex immunoperoxidase method) was used in both Western blotting and immunohistochemistry.8) Ha-ras p21 antibody was obtained from Triton Biosciences (Alameda, USA) and used at a concentra-
tion of 13.5 µg/ml in immunohistochemistry and 5 µg/ml in Western blotting. The antibody was generated in sheep against the synthetic peptide corresponding to positions 160-179 of v-Ha-ras p21 and purified by utilizing the immunogenic peptide-binding affinity.10)

The specificity of the antibody was examined by using Western blotting, for which normal rat kidney cells transformed by Harvey sarcoma virus (Ha-NRK) and Kirsten sarcoma virus (Ki-NRK) were used as a positive control for ras p21. Samples equivalent to 40 µg or 100 µg of protein were loaded onto 15% NaDOSO₄-polyacrylamide gel. After electrophoresis, they were electroblotted onto a nitrocellulose filter (0.45 µm pore size, Schleicher and Schuell, West Germany) and incubated with Ha-ras p21 antibody. An avidin-biotin system (Vectastain ABC kit, Vector Lab. Inc., USA) using a secondary anti-sheep IgG rabbit antiserum (diluted 1:150, vector Lab.) was used to visualize p21 bands. The specificity of the reaction was determined by competition assay as follows. The electroblotted nitrocellulose filter was incubated in a mixture of Ha-ras p21 antibody and about 100-fold molar excess of the antigenic synthetic peptide in the primary reaction. The antigenic peptide was kindly supplied by Dr. T. Tanaka,10) Department of Pediatrics, Hiroshima University School of Medicine, who purified the antibody used in this study.

In Ha-NRK cells, the v-ras gene product was detected as two bands of 21 kDa (Fig. 1a), due to phosphorylation of the viral p21 at the threonine residue at position 59, resulting in a more slowly migrating form of p21.11) No p21 band was found in Ki-NRK cells. The amino acid sequence of the N-ras product in the C-terminal region is similar to that of Ki-ras but different from that of Ha-ras. Furthermore, the synthetic peptide corresponding to residues 160-179 of v-Ha-ras used for raising the antibody is exactly the same as that of c-Ha-ras.12) Therefore, the antibody used was considered to recognize c-Ha-ras p21 as well as v-Ha-ras p21. The antigenic peptide competed with the p21 bands but the other bands were not affected (Fig. 1b). Most of the bands other than p21 were also detected when the primary reaction was omitted (not shown).

All of 4 gastric carcinomas had a single band of p21 and the level varied from case to case (Fig. 2). The antigenic peptide also competed with the p21 band in a gastric tumor (Fig. 1b). In nonneoplastic gastric mucosa, one band of p21 was detected in both areas (fundus and antrum) but the levels of p21 in gastric carcinomas were clearly
higher than that in nonneoplastic mucosa. The c-ras genes encode alanine at position 59 and this amino acid cannot be phosphorylated. No slowly migrating phosphorylated form of p21 was seen in either gastric tumors or nonneoplastic mucosa. The p21s detected in these tissues were considered to have originated from the c-Ha-ras gene. However, Ha-ras p21 antibody used could not distinguish between normal and activated c-Ha-ras p21. Two (No. 2965 and 2081) out of the aforementioned four gastric tumors showed immunohistochemical reaction to c-Ha-ras p21.

The specificity of immunohistochemical reaction was examined according to the method of Sternberger. When the antibody was absorbed with a 2-fold molar excess of the antigenic peptide at 4°C for 24 hr, the reaction product specifically disappeared (Fig. 3a, b). Positive immunoreactivity of c-Ha-ras was defined as reactivity which was absorbed with the antigenic peptide. The p21-immunoreactivity within the tumor tissue was graded as follows: tissue with more than 50% of p21-positive tumor cells was graded ++, 25% to 50% +, less than 25% +, and negative −.

![Image of immunohistochemical staining of gastric adenocarcinoma with Ha-ras p21 antibody.](image)

### Table I. Cases with c-Ha-ras p21-immunoreactivity in Tumor Cells of 171 Gastric Carcinomas

<table>
<thead>
<tr>
<th>Stage</th>
<th>Histological type</th>
<th>No. of cases</th>
<th>Cases with c-Ha-ras p21-immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>pap, tub</td>
<td>18</td>
<td>2 (2) 11.1 (11.1)</td>
</tr>
<tr>
<td>(27 cases)</td>
<td>por, sig</td>
<td>9</td>
<td>1 (1) 11.1 (11.1)</td>
</tr>
<tr>
<td>Advanced</td>
<td>pap, tub</td>
<td>49</td>
<td>30 (15) 61.2 (30.6)</td>
</tr>
<tr>
<td>(144 cases)</td>
<td>por, sig</td>
<td>63</td>
<td>22 (10) 34.9 (15.9)</td>
</tr>
<tr>
<td></td>
<td>sci</td>
<td>32</td>
<td>11 (9) 34.3 (28.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63 (34)</td>
<td>43.8 (23.6)</td>
</tr>
</tbody>
</table>

a) According to the classification of the Japanese Research Society for Gastric Cancer: pap, papillary adenocarcinoma; tub, tubular adenocarcinoma; sig, signet ring cell carcinoma; por, poorly differentiated adenocarcinoma; sci, scirrhous carcinoma.
b) Number of cases or incidence of cases showing ++ or +++ p21-immunoreactivity.
c) Total cases with p21-immunoreactivity, significantly different from early carcinoma and advanced carcinoma (P < 0.01).
c-Ha-ras p21-immunoreactivity within tumor cells of 171 gastric carcinomas is shown in Table I. The p21-positive cells were observed in 3 (11.1%) of the 27 early carcinomas and in 63 (43.8%) of the 144 advanced carcinomas, the incidence being significantly different (P<0.01). In advanced carcinomas, the incidence of p21-immunoreactivity grade ++ or +++ in well differentiated adenocarcinoma was higher than that in poorly differentiated adenocarcinoma but was not different from that in scirrhus carcinoma corresponding to Borrmann’s type 4 carcinoma or diffusely infiltrative carcinoma. The p21-positive tumor cells showed both cytoplasmic and surface membrane staining (Figs. 3a and 4).

In p21-immunoreactivity grade ++ or +++ cases, p21 was detected more frequently in deeply invasive carcinomas than in superficially invasive carcinomas, the incidence being 10 (66.7%) of the 15 well differentiated advanced carcinomas and 4 (44.4%) of the 9 scirrhus carcinomas.

Of the 144 advanced carcinomas, 50 cases showed metastases to perigastric lymph nodes. Out of the 50 metastatic tumor cases, 34 (68.0%) had p21-immunoreactivity which was distributed diffusely within metastatic tumors of perigastric lymph nodes (Fig. 4). The 34 cases accounted for 23 (85.2%) out of the 27 primary tumor-p21-positive cases and 11 (47.8%) out of the 23 primary tumor-p21-negative cases (Table II). The intensity and incidence of p21-immunoreactivity in the metastatic tumors were evidently greater than those in primary tumors (Fig. 4). In nonneoplastic gastric mucosa, parietal cells, regenerative epithelia and hyperplastic glands adjacent to tumors occasionally showed p21-immunoreactivity. After absorption with the antigenic peptide, these epithelial cells showed disappearance of the reaction product.

A follow-up study was made on 68 cases of stage III and stage IV advanced carcinomas, and the 3-year survival rates were compared for patients with tumors positive (immunoreactivity grade ++ or +++) and negative for c-Ha-ras p21. For 25 p21-positive carcinomas the 3-year survival rate was 6.9%, while that for 43 p21-negative carcinomas was 25.4% (Fig. 5). Patients with p21-positive carcinoma had a poorer prognosis than those with p21-negative carcinoma, and a significant difference was observed 15 months after gastrectomy (P<0.05).

In this study, the incidence of c-Ha-ras p21 expression was about 11% in early carcinomas and 44% in advanced carcinomas regardless of histological type. However,

Table II. Cases with c-Ha-ras p21-immunoreactivity in Tumor Cells of 50 Gastric Carcinomas Metastasizing to Perigastric Lymph Nodes

<table>
<thead>
<tr>
<th>c-Ha-ras p21-immunoreactivity in primary tumors</th>
<th>No. of cases</th>
<th>Cases with c-Ha-ras p21-immunoreactivity in metastatic tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>34</td>
</tr>
</tbody>
</table>

a) Total cases showing metastases to perigastric lymph nodes.
b) Total cases showing p21-immunoreactivity in metastatic tumors of perigastric lymph nodes.
Ha-ras p21 IN GASTRIC CANCER

it is of considerable interest that biological correlations evidently existed between the p21-immunoreactivity and invasion, metastasis and prognosis of human gastric carcinoma. c-Ha-ras p21-immunoreactivity within tumors was correlated with the depth of tumor invasion through the gastric wall. These findings are in agreement with the results of immunohistochemical observations on human colon carcinomas. Gallick et al. reported that the levels of c-Ha-ras expression in human colorectal carcinomas decreased in metastatic lesions. In this study, however, c-Ha-ras p21-immunoreactivity in metastatic foci was stronger than that in the primary tumor. In addition, the prognosis of patients with p21-positive carcinomas was apparently worse than that of patients with p21-negative cases. These results strongly suggest that c-Ha-ras p21 expression plays an important role in tumor invasion and metastasis of gastric carcinoma and that the abnormal expression of c-Ha-ras p21 serves as a biological marker of high malignancy in patients with gastric carcinoma.

Several explanations are possible for increased expression of ras p21 in metastasis of gastric cancer. Firstly, production of such growth factors as human epidermal growth factor (EGF) might enhance the expression of the c-ras family. For example, we have demonstrated that the expression of EGF in gastric carcinomas as well as c-Ha-ras p21 is closely correlated with the depth of tumor invasion and the prognosis, and increases in metastatic sites. Among EGF-positive metastatic carcinomas of perigastric lymph nodes, about 67% showed Ha-ras p21-immunoreactivity synchronously (unpublished data). EGF has been found to enhance the guanine nucleotide-binding activity of activated c-Ha-ras or v-Ha-ras p21 in isolated membranes. Secondly, other oncogenes, such as the myc family, which has been shown to be amplified in human gastric carcinoma, might influence expression of the c-ras gene. Thirdly, ras p21 might act similarly to GTP-binding protein coupling in peptide hormone-adenyl cyclase interactions. Gastrin, which has a trophic action on gastrointestinal mucosa, has been shown to promote the growth of human gastric carcinoma through influencing cyclic AMP metabolism.

In view of the recent findings that EGF receptor has a close sequence homology with erythroblastosis virus oncogene protein (erb-B) and that gastrin is related to polyoma virus middle T protein, the interaction between EGF or polypeptide hormones/receptor systems and ras oncoprotein is considered to be important to our understanding of the role of the ras gene in tumor progression and malignancy of human gastric cancer.

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