A tumour-associated cell-surface glycoprotein accompanying p53 overexpression and higher growth potential for gastric cancer

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Summary Tumour-associated cell-surface glycoprotein is associated with tumour progression in gastric cancer. We investigated the biological significance of tumour-associated cell-surface glycoprotein, determined by the binding of *Helix pomatia* agglutinin (HPA), with regard to survival time and to the malignant potential of cancer cells in serosally invasive gastric cancer in 119 patients. HPA was positively stained in 75 of 119 patients (63.0%) with gastric cancer with serosal invasion. In patients with HPA-positive tissue, the tumour was larger than in HPA-negative cases and was frequently located in the middle third of the stomach. The incidence of lymph node metastasis was higher than in patients with HPA-negative tissue. There were no differences between the cases staining negatively and positively with HPA with respect to the other factors examined. Gastric cancer tissues with HPA-positive staining revealed a higher positive rate of abnormal p53 staining and a higher concentration of proliferating cell nuclear antigen (PCNA) labelling. The survival time of the patients with HPA positive staining was shorter than for those whose tissues were HPA negative. Thus, tumour-associated cell-surface glycoprotein is apparently closely related to the malignant potential of serosally invasive gastric cancer.

Keywords: gastric cancer; tumour-associated cell-surface glycoprotein; p53 overexpression; proliferating cell nuclear antigen; prognosis

Lectins are proteins of plant or animal origin which bind specifically to glycoconjugates. Using labelled lectin, carbohydrate residues in tissue sections can be detected using histochemical procedures. This has led to a search for lectin binding to detect carbohydrates related to tumour metastatic behaviour (Schumacher *et al.*, 1992), since cell-surface glycoproteins have important roles in cell-to-cell interaction, invasion and metastatic potential (Dennis *et al.*, 1987; Buckley and Carlsen, 1988; Olden, 1993). We have suggested that binding sites for a lectin from the Roman snail, *Helix pomatia* agglutinin (HPA), is a predictive marker of tumour progression in gastric cancer (Kakeji *et al.*, 1991a). HPA, with a molecular mass of 79 kDa and a strong affinity for *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine, specifically agglutinates human type A erythrocytes (Kawai *et al.*, 1991). In particular, the presence of lymph node metastasis is related to HPA binding of gastric cancer cells. The intracellular events linked to these antigens expressed on the surface of gastric cancer cells have remained unresolved.

At the level of the nucleus, abnormalities of tumour-related genes and growth potential have been reported to relate to the poor prognosis of the patient (Mori *et al.*, 1993; Uchino *et al.*, 1993). The p53 gene is a tumour-suppressor gene and p53 is a nuclear protein, a transcriptional activator which regulates the onset of DNA replication at the $G_1 - S$ boundary (Vogelstein *et al.*, 1992). The great majority of mutations in the p53 gene are missense in type, leading to production of full-length proteins with an altered conformation and abnormal biological properties (Hollstein *et al.*, 1991). Unlike the wild-type protein, which has a short half-life, the great majority of tetramers containing mutant protein are stable and therefore readily detectable by immunochemistry using antibodies reactive to p53 (Kikuchi-Yanoshita *et al.*, 1992).

The primary aim of the present study was to determine whether there was a relationship between the glycoproteins and cell growth and, if so, what would be the effect on biological aggressiveness of the cancer. Serosally invasive gastric cancer has a variety of forms of recurrence and is associated with a poorer prognosis than those with a lesser

degree of invasion. For such patients, we asked whether the abnormality of cell-surface glycoproteins is associated with the malignant potential and proliferating activity of the cancer cells, determined by the concentration of proliferating cell nuclear antigen (PCNA) labelling.

Patients and methods

This study included 119 unselected patients with primary gastric cancer with serosal invasion, all of whom underwent gastric resection in the Department of Surgery II, Kyushu University, and affiliated hospitals in Fukuoka, Japan, from 1988 to 1991. All were examined clinically and pathologically with respect to the factors given in Table I. Pathological diagnosis and classification of the resected gastric cancer tissues were made according to the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan (Japanese Research Society for Gastric Cancer, 1981, 1993; Maehara *et al.*, 1992). Gastric resection based on lymph node dissection was classified as follows: D0, gastric resection, including the incomplete removal of group 1 lymph nodes; D1, gastric resection, including the complete removal of group 1 lymph nodes alone; D2, gastric resection including the complete removal of group 1 and 2 lymph nodes; and D3, gastric resection, including the complete removal of group 1, 2 and 3 lymph nodes.

Tissue samples

Tissue samples for p53 staining with monoclonal antibody PAB 240 were fixed in periodate–lysine–paraformaldehyde (PLP) for 5 h immediately after surgical resection, embedded in OTC compound (Miles, Elkart, IN, USA), embedding medium for frozen tissue specimens, and preserved at − 80°C (Kakeji *et al.*, 1993). Sections 5 μm thick were cut on a cryostat. For PCNA staining, paraffin blocks were prepared as described by Mori *et al.* (1993).

Lectin histochemistry

Sections of 5 μm from paraffin blocks were dewaxed and stained using the avidin–biotin–peroxidase complex (ABC) method. Specifically, sections were incubated for 1 h with HPA (10 μg ml⁻¹; Pharmacia, Sigma, St Louis, MO, USA) at

Table I Clinicopathological factors (mean ± s.d.) in serosally invasive gastric cancers with or without lymph node metastasis

Factor	HPA-negative (n = 44)	HPA-positive (n = 75)	P-value
Age (years)	61.1 ± 11.9	60.4 ± 13.2	NS
Sex			
Male	25 (56.8%)	49 (65.3%)	NS
Female	19 (43.2%)	26 (34.7%)	
Maximum tumour diameter (cm)	8.20 ± 4.08	9.58 ± 3.72	P<0.05
Tumour location			
Upper (C)	15 (34.1%)	16 (21.3%)	P<0.05
Middle (M)	9 (20.5%)	36 (48.0%)	
Lower (A)	20 (45.4%)	23 (30.7%)	
Histology			
Differentiated	19 (45.2%)	34 (45.9%)	NS
Undifferentiated	23 (54.8%)	40 (54.1%)	
Specific[a]	1	1	
Unknown[a]	1	0	
Histological growth pattern			
Expansive	5 (11.4%)	6 (8.2%)	NS
Intermediate	18 (0.9%)	24 (32.9%)	
Infiltrative	21 (47.7%)	43 (58.9%)	
Unknown[a]	0	2	
Lymphatic involvement			
No	9 (20.9%)	8 (10.7%)	NS
Yes	34 (79.1%)	67 (89.3%)	
Unknown[a]	1	0	
Vascular involvement			
No	19 (44.2%)	42 (59.2%)	NS
Yes	24 (55.8%)	29 (40.8%)	
Unknown[a]	1	4	
Lymph node metastasis			
No	12 (28.6%)	8 (11.0%)	P<0.05
Yes	30 (71.4%)	65 (89.0%)	
Unknown[a]	2	2	
Peritoneal dissemination			
No	40 (90.9%)	65 (86.7%)	NS
Yes	4 (9.1%)	10 (13.3%)	
Liver metastasis			
No	41 (93.2%)	65 (86.7%)	NS
Yes	3 (6.8%)	10 (13.3%)	
Gastric resection			
Partial	19 (43.2%)	30 (40.0%)	NS
Total	25 (56.8%)	45 (60.0%)	
Lymph node dissection			
D1	8 (18.2%)	15 (20.0%)	NS
D2 and D3	36 (81.8%)	60 (80.0%)	
Curability			
Curable	31 (70.5%)	50 (66.7%)	NS
Non-curable	13 (29.5%)	25 (33.3%)	

NS, no significant difference. [a]These cases were excluded from statistical analysis.

room temperature before adding, sequentially, rabbit anti-serum to HPA (1:10 for 1 h; Serotec, Oxford, UK) and biotinylated goat anti-rabbit IgG (1:400 for 30 min; Bethesda Research Laboratories, Gaithersburg, MD, USA) (Kakeji et al., 1991a). The tissues were stained for 30 min using ABC kits (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine/hydrogen peroxidase. Sections were counterstained with Mayer's haematoxylin. using a microscopic grid, the percentage of positively stained tumour cells was calculated and graded according to the criteria of Kakeji et al. (1991a), as follows: negative staining, 0–10%; positive staining, more than 11%.

Immunohistochemical staining of p53

PAb 240 is a mouse monoclonal antibody that recognises an evolutionarily conserved epitope on p53; the epitope lies between amino acids 156 and 214 on murine p53 (Gannon et al., 1990). This antibody reacted with human, mouse, rat, hamster, bovine and chicken p53 in Western immunoblotting experiments. Sections of 5 μm were cut on a cryostat, dried and washed in phosphate-buffered saline, pH 7.2 (PBS), then incubated at room temperature with normal horse serum (1:10 for 15 min; Vector Laboratories). We then added PAb 240 (1:50 overnight; Oncogene Science, NY, USA), biotinylated horse anti-mouse IgG (1:200 for 30 min; Vector Laboratories) and the avidin–biotin–peroxidase complex (for 30 min; Vector Laboratories). Peroxidase labelling was developed with 3,3'-diaminobenzidine and hydrogen peroxidase and the sections were counterstained with haematoxylin. We used KUPL40 as the positive control, a carcinoma from a patient with familial polyposis coli transplanted into nude mice and with a mutation in the p53 gene, as detected by sequencing. Omission of the primary antibody served as the negative control.

Proliferating cell nuclear antigen (PCNA) staining

Sections of 5 μm from paraffin blocks were dewaxed in xylene, rehydrated through a series of ethanol and immersed in 0.3% (v/v) hydrogen peroxide in methanol. These sections were subsequently washed in PBS, and normal goat serum was applied to reduce non-specific binding. The primary antibody PC10, a monoclonal mouse antibody for rat PCNA, was purchased from Dako (Denmark). The sections were incubated for 2 h with PC10 (dilution 1:20) at room temperature, then with biotinylated goat anti-mouse IgG (1:200 for 1 h), and finally with the avidin–biotin–peroxidase complex (Mori et al., 1993). Peroxidase labelling was developed with 3,3'-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with haematoxylin. All stained nuclei were scored as positive for PCNA. The PCNA labelling index was determined by observing 1000 nuclei in areas of the section with the highest labelling frequency, and the percentage of PCNA-labelled nuclei (PCNA labelling index) was used for analysis.

Statistical analysis

The BMDP Statistical Package program (BMDP; Los Angeles, CA, USA) for the IBM (Armonk, NY, USA) 4381 mainframe computer was used for all analyses (Dixon, 1988). The BMDP P4F and P3S programs were used for the chi-square test and the Mann–Whitney test to compare data on patients between the groups. The BMDP P1L program was used to analyse survival time by the Kaplan–Meier method and the Mantel–Cox test was used to test for equality of the survival curves. The accepted level of significance was P<0.05.

Results

Clinicopathological factors

HPA-positve cells showed either strong surface staining or, in some cases, apical or diffuse cytoplasmic staining. In normal tissue, positive staining with HPA was recognised in mucous neck cells and in pyloric glands. HPA was also noted in the basement membrane of small blood vessels in the cases examined. In cancer tissues, positive HPA staining was demonstrated in 75 (63.0%) of 119 primary tumours. No obvious relation was found between HPA staining and the sex or age of the patient (Table I). HPA-positive isolates were associated with a larger tumour and location in the middle third of the stomach and a higher incidence of metastasis to lymph nodes (89.0%) than were HPA-negative cases (71.4%) (P<0.05). The incidence of HPA staining was not related to tumour histological type, lymphatic and venous invasion, peritoneal dissemination, or liver metastasis for serosally invasive gastric cancer.

Table II Relation between the grouping for HPA staining and p53 staining in gastric cancer tissues

Factor	HPA-negative	HPA-positive	P-value
p53-negative	30 (76.9%)	18 (31.0%)	P<0.01
p53-positive	9 (23.1%)	40 (69.0%)	
Total	39 (100%)	58 (100%)	

Table III Growth potential evaluated by PCNA labelling in gastric cancer invading beyond the serosa

Factor	HPA-negative (n = 25)	HPA-positive (n = 51)	P-value
PCNA-labelling (%)	26.9 ± 12.3[a]	38.5 ± 10.2	P<0.01

[a]Mean ± standard deviation.

Table IV Site of recurrence after resection for gastric cancer invading beyond the serosa

Site of recurrence	HPA-negative	HPA-positive
Peritoneum	7 (38.9%)	20 (35.7%)
Liver	1 (5.6%)	6 (10.7%)
Lung	2 (11.1%)	4 (7.1%)
Bone	0	2 (3.6%)
Brain	1 (5.6%)	2 (3.6%)
Local	2 (11.1%)	6 (10.7%)
Lymph node	2 (11.1%)	5 (8.9%)
Unknown	5 (27.8%)	18 (32.1%)
Total	18 (100%)	56 (100%)

Figure 1 Survival curves for patients with HPA negative and HPA positive gastric cancer tissues. Patients with HPA-positive tissues (thin line) had a poorer prognosis than those for HPA-negative ones (thick line) (P<0.01).

Relation between HPA and p53

We determined the relation between the HPA staining and abnormal p53 staining. The positive rate for p53 was 23.1% (9/39) in HPA-negative patients and 69.0% (40/58) in HPA-positive patients, with a significant difference (P<0.01) (Table II).

HPA staining and proliferating activity

The proliferating activity, expressed by PCNA labelling, was significantly higher in tumour tissues with HPA-positive staining than in those with HPA-negative staining, with a significant difference (P<0.01) (Table III).
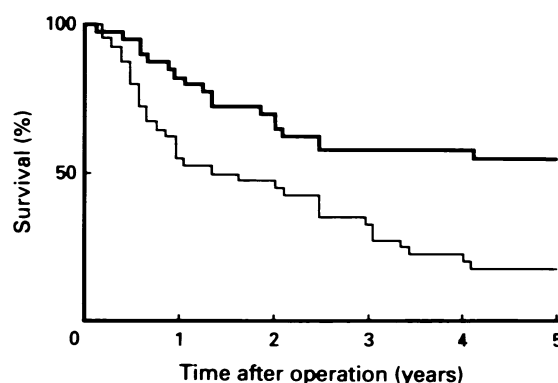
Recurrence pattern

The rate of recurrence was 40.9% (18/44) for HPA-negative patients and 74.7% (56/75) for HPA-positive ones (Table IV). Peritoneal and local recurrences were mainly noted in HPA-negative patients, while there was a variety of forms of recurrences, including peritoneal and distant organ recurrences in the HPA-positive patients.

Survival rate

The overall survival curve is shown in Figure 1, according to HPA-negative or -positive staining. Patients with HPA-positive cancers had a shorter survival time than did those with HPA-negative cancers (P<0.01). The 5 year survival rate was 54.4% for HPA-negative patients and 18.0% for HPA-positive ones.

Discussion

Cellular interactions and the rate of growth of cancer cells seem to have an important role in the clinical behaviour of tumours (Hakomori et al., 1989; Lampe et al., 1993). These interactions are partly mediated by cell-surface glycoproteins which are closely associated with the neoplastic transformation of cells (Altevogt et al., 1983; Pierce and Arango, 1986).

Changes in cell-surface glycoproteins are thought to be associated with altered cell adhesion and with the development of invasive and metastatic properties in experimental and human tumours (Dennis et al., 1987; Buckley and Carlsen, 1988; Dennis and Laferte, 1989; Sell, 1990). Changes in cell-surface carbohydrate occur during tumour progression, and these changes can be detected by studying the binding patterns of lectins (Hiraizumi et al., 1990). Positive staining for HPA, which recognises N-acetyl-D-galactosamine and N-acetyl-D-glucosamine, was noted in cases of breast cancer to be associated with metastasis to local lymph nodes and with a higher recurrence and mortality rate (Fukutomi et al., 1989). We have reported that the altered glycosylation of cell-surface glycoprotein in gastric cancer, which was recognised by HPA, is associated with lymph node metastasis and a higher mortality rate, and we suggested that HPA staining of the gastric cancer is closely related to the potential for lymphatic spread of gastric cancer (Kakeji et al., 1991a). These data prompted us to determine the molecular events related to HPA-positive gastric cancers.

A close relation between HPA staining and amplification of the c-myc gene or c-erbB-2 gene has been noted in human breast cancer tissues (Fukutomi et al., 1991; Thomas et al., 1993). In the present study, we clarified the correlations between HPA staining of cancer cells, abnormality of the p53 gene and the proliferating activity determined by PCNA labelling in gastric cancer. Carder et al. (1993) stated that cells containing abnormal p53 protein are particularly at risk of endoreduplication and hence of tetraploidy. Failure to regulate p53 expression may lead to uncontrolled cell growth (Lane, 1992; Livingstone et al., 1992; Yin et al., 1992). The concentrations of PCNA, a highly conserved 36 kDa acidic protein and auxiliary protein for DNA polymerase δ, which is directly involved in DNA synthesis (Hall et al., 1990; Waseem and Lane, 1990), correlate with the proliferative state of cells and with the prognosis in gastric cancer (Mori et al., 1993). PCNA labelling correlates with other parameters of cell proliferation (Ki-67 score, S-phase fraction, as determined by flow cytometry, bromodeoxyuridine and thymidine incorporations into DNA, and mitotic index) and with DNA ploidy in human tumours (Hall et al., 1990; Kakeji et al., 1991b). Thus, intracellular events related to p53 abnormality and detected by p53 protein staining are linked to increased cellular proliferation and to the phenotype of altered cell membrane glycoproteins. We have reported that overexpression of p53 is closely related to the potential of cancer cells to metastasise to lymph nodes in gastric cancer (Kakeji et al., 1993). Joypaul et al. (1994) reported that p53 overexpression is an independent marker of a shorter survival for gastric cancer patients. The p53 abnormality is likely to accelerate the lymphatic spread of cancer cells through association with altered cell membrane glycoproteins and a poorer prognosis.

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The results of this study suggest that abnormalities in cell-surface glycoproteins are closely linked to an abnormal p53 gene and to a higher growth potential in gastric cancer. These prognostic factors are assumed to be related to the aggressiveness of gastric cancer. As tumours with high proliferative activity are sensitive to anti-cancer drugs (Maehara

et al., 1990), HPA staining may aid in designing treatment strategies for particular groups of patients.

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