

ORIGINAL ARTICLE

Pathobiological characteristics of intestinal and diffuse-type gastric carcinoma in Japan: an immunostaining study on the tissue microarray

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Aim: To investigate the pathobiological features of intestinal and diffuse-type gastric carcinomas in the Japanese population.

Methods: The expression of fragile histone triad (FHIT), phosphatase and tensin homology deleted from human chromosome 10 (PTEN), caspase-3, Ki-67, mutant p53, matrix metalloproteinase (MMP)-2, MMP-9, and extracellular matrix metalloproteinase inducer (EMMPPRIN) on tissue microarrays of gastric carcinomas by immunostaining was examined in comparison with the clinicopathological characteristics between intestinal and diffuse-type cases.

Results: Intestinal-type carcinoma frequently occurred in old men, whereas the diffuse type comparatively occurred more in young women ($p < 0.05$). The diffuse-type carcinoma was more inclined to invasion into muscularis propria, lymphatic invasion and lymph node metastasis, and belonged to higher International Union against Cancer (UICC) staging ($p < 0.05$) compared with intestinal-type counterparts. Expression of FHIT, PTEN, Ki-67, caspase-3, mutant p53 and EMMPPRIN was higher in intestinal-type carcinomas than in diffuse-type carcinomas ($p < 0.05$). Kaplan–Meier analysis indicated that patients with intestinal-type carcinomas had a higher cumulative survival rate ($p < 0.05$).

Conclusion: Intestinal-type gastric carcinomas with a more favourable prognosis frequently show high levels of proliferation and apoptosis, and always accompany strong expression of FHIT, PTEN and mutant p53 and EMMPPRIN. EMMPPRIN expression might underlie the molecular basis of liver metastasis and higher proliferation of intestinal-type gastric carcinomas in Japan. Lauren's classification thus proved pathologically relevant for the clinical treatment of gastric carcinomas.

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Gastric carcinoma is ranked as the world's second leading cause of mortality due to cancer next to lung cancer, despite a sharp worldwide decline in both its incidence and mortality since the second half of the 20th century.¹ It continues to be a major health problem because of the slow decrease in incidence in Asia and high mortality of diagnosed gastric carcinoma in West.² Generally, prognosis of the patients with gastric carcinoma is dependent on its histological type. Therefore, it is of general importance to establish a practical and reliable histological classification of gastric carcinoma for clinical and pathological work.

Although gastric carcinoma is a malignant tumour originating from the same gastric epithelium, its morphological features vary substantially with the individual patients. As a result, there are many histological classifications based on morphological features. According to the World Health Organization (WHO),³ gastric carcinomas are mainly classified into papillary adenocarcinoma, well-differentiated, moderately-differentiated or poorly-differentiated adenocarcinoma, mucinous adenocarcinoma, signet ring cell carcinoma (SRC) and undifferentiated carcinoma. The classification only emphasises the morphological appearance on gastric carcinoma and therefore is not suitable for clarifying the questions of histopathogenesis. Based on the criteria proposed by Nakamura,⁴ gastric carcinomas are divided into differentiated and undifferentiated carcinomas, but it is very difficult to classify the moderately differentiated and mucinous adenocarcinoma using this approach. This dilemma demands a simple, practical and convenient classification to explore the histological architecture of gastric carcinoma, such as intestinal-type and diffuse-type

division advocated by Lauren.⁵ Histologically, the intestinal types principally include papillary, well-differentiated adenocarcinoma, moderately differentiated or mucinous adenocarcinoma without SCR cells, whereas diffuse types mainly consist of poorly differentiated adenocarcinoma, SRC carcinoma and undifferentiated adenocarcinoma of WHO classification. Although approximately 15% of gastric carcinomas are characterised as unclassified or mixed type, an intermediate type of gastric carcinoma may possibly have a few special changes, reflecting polyclonal histogenesis and being aggressive in histological differentiation with progression.

Histologically significant differences between gastric carcinomas may be mainly attributed to the originating cells, especially the genetic changes in these cells. To explore the clinicopathological features of intestinal-type and diffuse-type carcinomas in the Japanese population, we examined the proliferative or apoptotic levels of carcinoma cells by Ki-67 and caspase-3 immunostaining using a tissue microarray approach, along with assessment of the expression of some tumour suppressor genes (TSG), such as like fragile histone triad (FHIT), phosphatase and tensin homology deleted from human chromosome 10 (PTEN), p53, matrix metalloproteinase (MMP) or its regulator, including MMP-2, MMP-9 and extracellular matrix metalloproteinase inducer (EMMPPRIN).

Abbreviations: EMMPPRIN, extracellular matrix metalloproteinase inducer; FHIT, fragile histone triad; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homology deleted from human chromosome 10; SRC, signet ring cell carcinoma; TMA, tissue microarray; TSG, tumour suppressor gene; WHO, World Health Organization

MATERIALS AND METHODS

Subjects

A total of 316 cases of gastric carcinomas were collected from our affiliated hospital and related institutes between 1993 and 2002. The patients with gastric carcinoma included 222 men and 94 women (mean age 66.2 years; range 38–88 years). Among them, 129 patients presented with lymph node metastasis and 15 with liver metastasis. None of these cases underwent chemotherapy or radiotherapy before surgery. All provided consent for use of tumour tissue for clinical research, and our University Ethical Committee approved the research protocol. We followed up all patients by consulting their case documents and by telephone.

Pathology

All tissues were fixed in 4% neutralised formaldehyde, embedded in paraffin wax and incised into 4-mm sections. These sections were stained by haematoxylin and eosin to confirm their histological diagnosis and other microscopic characteristics. The staging for each gastric carcinoma was evaluated according to the International Union against Cancer system, indicating the extent of tumour spread.⁶ Histomorphological architecture of the tumours was expressed according to Lauren's classification.⁵ Furthermore, depth of invasion, lymphatic invasion and lymph node metastasis were determined as well.

Tissue microarray

In all, 247 patients with gastric carcinomas were randomly selected for tissue microarray (TMA). Haematoxylin and eosin stained sections of the selected tumours were examined and representative areas of solid tumour identified for sampling. A tissue core of diameter 2 mm was punched off from each donor block and transferred to a recipient block of maximum 48 cores using Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan). Sections of 4 µm thickness were consecutively incised from the recipient block and transferred to poly-lysine-coated glass slides. Haematoxylin and eosin staining was performed on TMA for the confirmation of tumour samples (fig 1A).

Immunohistochemistry

Consecutive sections were deparaffinised with xylene, dehydrated with alcohol and subjected to antigen retrieval by irradiating in target retrieval solution (DAKO, Carpinteria, California, USA) for 5 min with microwave oven (Oriental Rotor, Tokyo, Japan). Bovine serum albumin 5% was then applied for 1 min to prevent non-specific binding. The sections were incubated with primary antibodies for 20 min, then treated with the anti-mouse or anti-rabbit Envision-PO (DAKO) antibodies for 20 min. All incubations were performed in the microwave oven for intermittent irradiation as described previously.⁷ After each treatment, the slides were washed with TRIS-buffered NaCl solution with Tween (10 mM TRIS-HCl, 150 mM NaCl, 0.1% Tween 20) three times for 1 min. Table 1 summarises the first antibodies. All slides were coloured with 3,3'-diaminobenzidine and counterstained with Mayer's haematoxylin. Omission of the primary antibody was used as a negative control and appropriate positive controls were used as recommended by the manufacturers.

Evaluation of immunohistochemistry

The immunoreactivity to FHIT, caspase-3, MMP-2 and MMP-9, was localised in the cytoplasm, PTEN, Ki-67 and mutant p53 only in the nucleus, and EMMPRIN in the cytoplasm and membrane (fig 1B–I). One hundred cells were randomly selected and counted from five representative fields of each section blindly by two independent observers. The positive

percentage of counted cells was graded as negative (0–5%) and positive (6–100%).

Statistical analysis

Statistical evaluation was performed using Fisher's exact possibility test to differentiate the rates, and using Mann–Whitney U test to differentiate the means of different groups. Kaplan–Meier survival plots were generated and comparisons between survival curves were made using the log-rank statistic. $p < 0.05$ was considered as significant. SPSS V.10.0 software was used to analyse all data.

RESULTS

Compared with intestinal-type gastric carcinoma, diffuse-type lesions were frequently encountered in young women and were prone to invasion into muscularis propria, lymphatic invasion and lymph node metastasis ($p < 0.05$). Most of them belonged to higher International Union against Cancer staging ($p < 0.05$; table 2). As summarised in table 2, expression of FHIT, PTEN, Ki-67, caspase-3, mutant p53 and EMMPRIN was higher in intestinal-type than in diffuse-type carcinomas ($p < 0.05$), whereas there was no difference in expression of MMP-2 or MMP-9 between the two ($p > 0.05$).

Follow-up information was available on 316 patients with gastric carcinoma for a period ranging from 0.2 months to 12.2 years (mean, 27 months). Figure 2 shows the survival curves stratified according to Lauren's classification. Kaplan–Meier analysis indicated that the patients with intestinal-type carcinoma had higher cumulative survival rate than those with diffuse-type lesion ($p < 0.05$).

DISCUSSION

Histologically, Lauren's classification of gastric carcinomas is based on the morphological appearance. Intestinal-type carcinomas are characterised by cohesive carcinoma cells forming gland-like tubular structures with expanding or infiltrative growth pattern, such as papillary and well-differentiated adenocarcinoma.² However, the cell cohesion is less apparent or absent in diffuse-type carcinoma and cancer cells diffusely spread in the gastric wall as poorly differentiated adenocarcinoma, SRC and undifferentiated carcinoma. Generally, intestinal-type carcinomas originate from the atrophic gastritis or intestinal metaplasia of gastric epithelium, the latter being considered as an important precancerous lesion.⁸ Diffuse-type carcinomas are derived from the stem cells of gastric gland neck. Although a recent study indicated that all gastric epithelial cells originated from the stem cells of bone marrow,⁹ malignant transformation takes place in a comparatively mature stage of gastric epithelial cells for intestinal-type carcinoma.

Our study showed that intestinal-type carcinoma was common in the old men, whereas diffuse-type carcinoma was comparatively frequent in young women, consistent with Lauren's report.⁵ Men are more likely to drink alcohol or smoke, which could cause precancerous lesions such as intestinal metaplasia or dysplasia, closely correlated with the genesis of intestinal-type carcinoma.⁸ The fact that young women might have diffuse-type carcinomas without a long precancerous stage suggests that the contribution of genetic factors is higher than environmental factors with this type of carcinoma. Clearly, the distributions of sex and age in both kinds of carcinoma are of great significance for screening and prevention of gastric neoplasia.

We also found that diffuse-type carcinomas are more malignant than their intestinal-type counterparts, with early invasion into the muscularis propria and the lymphatic vessel, and metastasis to the lymph node. Zhang *et al.*¹⁰ reported SRCs

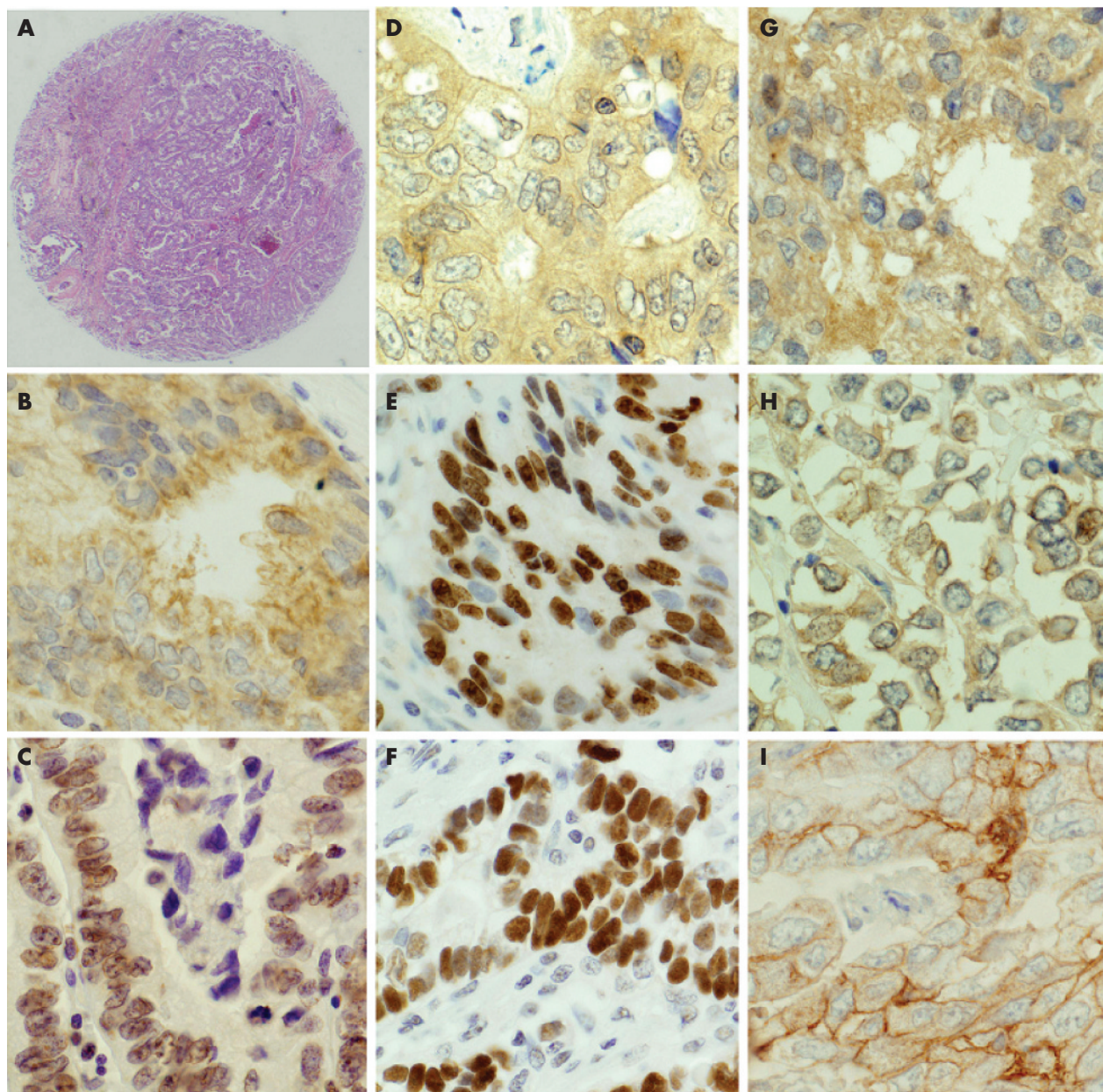


Figure 1 Haematoxylin and eosin staining and immunostaining in gastric carcinoma. Haematoxylin and eosin staining (A) on the tissue microarray of gastric carcinoma. The immunoreactivity to fragile histine triad (B), caspase-3 (D), matrix metalloproteinase (MMP)2 (G) and MMP-9 (H) was localised in the cytoplasm of gastric carcinoma cells, phosphatase and tensin homology deleted from human chromosome 10 (C), ki-67 (E) and mutant p53 (F) only in the nucleus, and in extracellular matrix metalloproteinase inducer (I) in the cytoplasm and membrane.

Names	Source	Company	Dilution
FHIT	Rabbit	Labvision, Fremont, USA	1:300
PTEN	Mouse	NovoCastr, Newcastle Upon Tyne, UK	1:150
Ki-67	Rabbit	DAKO, USA	1:25
Mutant p53	Mouse	DAKO, Carpinteria, USA	1:100
MMP-2	Mouse	Daiichi Fine Chemical, Toyama, Japan	1:50
MMP-9	Mouse	Daiichi Fine Chemical, Japan	1:150
EMMPRIN	Mouse	NovoCastr, UK	1:50

EMMPRIN, extracellular matrix metalloproteinase inducer; FHIT, fragile histine triad; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homology deleted from human chromosome 10

Table 2 Clinicopathological features of intestinal or diffuse type gastric carcinomas in Japanese population

Clinicopathological features	Intestinal-type carcinoma	Diffuse-type carcinoma
Histological types according to WHO classification	Papillary and tubular adenocarcinoma; part of moderately differentiated adenocarcinoma	Poorly differentiated or undifferentiated adenocarcinoma, SRC
Age (years), mean (SD)	67.8 (10.9), n = 163*	64.5 (10.5) n = 153
Sex (female:male)	1:3.4*	1:1.7
Invasion into muscularis propria	53/163 (32.5%)*	103/153 (67.3%)
Lymph node metastasis (+)	45/163 (27.6%)*	84/153 (54.9%)
Lymphatic invasion (+)	42/163 (25.8%)*	74/153 (48.4%)
UICC staging (O, I)	121/163 (74.2%)*	70/153 (45.8%)
Median survival time (months), mean (SD)	82.0 (20.5), n = 163*	22.9 (7.9) n = 153
FHIT expression (+)	98/132 (74.2%)*	64/110 (58.2%)
PTEN expression (+)	108/135 (80.0%)*	72/114 (63.2%)
Caspase-3 expression (+)	107/127 (84.3%)*	76/111 (68.5%)
Ki-67 expression (+)	127/132 (96.2%)*	104/114 (91.2%)
Mutant p53 expression (+)	112/135 (83.2%)*	76/108 (70.4%)
MMP-2 expression (+)	109/126 (86.5%)	90/101 (89.1%)
MMP-9 expression (+)	94/133 (70.7%)	73/110 (66.4%)
EMMPRIN expression (+)	88/124 (71.0%)*	64/111 (57.6%)

EMMPRIN, extracellular matrix metalloproteinase inducer; FHIT, fragile histone triad; IUCC, International Union against Cancer; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homology deleted from human chromosome 10; SRC, signet ring cell carcinoma; WHO, World Health Organization.

*Compared with diffuse-type carcinoma, $p < 0.05$.

to metastasise to the peritoneum via the migratory cancerous embolus of lymphatic vessel, and this might explain the inclination of diffuse-type carcinoma to invade and metastasise lymphatically, as well as spread peritoneally, as described by Wu *et al.*¹¹ Although the intestinal-type carcinoma readily metastasised to the liver, the biggest organ with biologically complicated functions,¹² the incidence of liver metastasis in our series was actually low. Consequently, intestinal carcinoma was closely linked to a favourable prognosis, as reported in this study. The poor prognosis with diffuse-type carcinoma is possibly due to its diffusely-invasive growth pattern, which makes it difficult for surgeons to macroscopically ensure a cancer-free margin. The other explanation is that diffuse-type gastric carcinoma easily metastasises via lymphatic vessels to the lymph node or peritoneally disseminates with high frequencies. Hence, this difference in pathological behaviours of both types of Lauren's classification is of practical significance in decision making about appropriate treatment options.

To clarify the pheno/genotypes of gastric carcinoma, we examined the expression of FHIT, PTEN, Ki-67, caspase-3 and mutant p53 by immunostaining. FHIT, PTEN and p53 have

important roles in regulating the balance between the proliferation and apoptosis as tumour suppressor gene.¹³⁻¹⁵ Ki-67 antigen is present in the nuclei of cells undergoing the proliferation, and should be regarded as a good marker for cell proliferation.¹⁶ Caspase-3 is responsible for the cleavage of poly(ADP-ribose) polymerase, actin and sterol regulatory element binding protein, and could reflect the apoptotic level in some sense as a key protease in cascade reaction of apoptotic pathway.¹⁷ Our Ki-67 and caspase-3 immunostaining results indicate that the intestinal-type carcinomas had high levels of proliferation and apoptosis compared with diffuse-type carcinoma in line with previous reports.¹⁸⁻¹⁹ Additionally, we found that there was higher expression of FHIT, PTEN and mutant p53 in intestinal-type than in diffuse-type carcinomas, suggesting that these molecules are mechanistically involved.

To explore the metastatic mechanisms of gastric carcinoma, we examined expression of MMPs and EMMPRIN, and found EMMPRIN to be more frequently expressed in the intestinal-type carcinoma, suggesting that EMMPRIN expression might be used as a good marker to differentiate between both kinds of carcinomas. A great deal of evidence indicates that increased EMMPRIN released from cancer cells could stimulate the MMP and VEGF expression of surrounding stromal cells, and that EMMPRIN transfection of tumour cells or treating tumour cells with the recombinant protein also increased the expression of MMPs, especially MMP-2.²⁰⁻²¹ Hence, we speculate that differential EMMPRIN expression might explain why intestinal-type gastric carcinoma easily metastasises to the liver. It is reported that EMMPRIN expression renders cancer resistant to apoptosis and stimulates hyaluronan production to sustain the anchorage-independent growth.²²⁻²³ Consequently, it is possible that higher EMMPRIN expression in intestinal-type gastric carcinomas may underlie the molecular basis of their higher proliferation.

In summary, intestinal-type carcinoma, which was positively correlated with favourable prognosis, frequently displayed high levels of proliferation and apoptosis and always accompanied expression of FHIT, PTEN and mutant p53 in Japan. EMMPRIN expression might contribute to the liver metastasis and proliferation of intestinal-type gastric carcinoma. Furthermore, our results confirm that Lauren's classification is relevant regarding histopathogenesis and differentiation, and as a guide to the clinical treatment of gastric carcinoma.

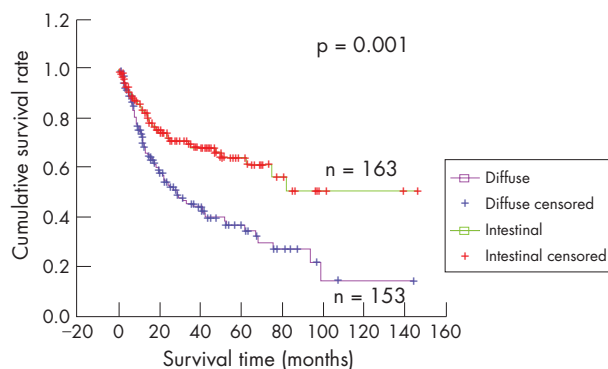


Figure 2 Relationship between Lauren's classification and prognosis of patients with gastric carcinoma. Kaplan-Meier curves of cumulative survival rate for the patients with gastric carcinoma according to the gastric histological classification of Lauren.

Take-home messages

- Intestinal-type carcinoma is common in old men, whereas diffuse-type carcinoma is comparatively frequent in young women.
- Diffuse-type carcinoma with worse prognosis is more malignant than its intestinal-type counterpart, with early invasion into the muscularis propria and the lymphatic vessel, and frequent metastasis to the lymph node.
- Compared with diffuse-type carcinoma, intestinal-type carcinoma exhibits high levels of proliferation and apoptosis, which are closely linked to high expression of fragile histone triad, phosphatase and tensin homology deleted from human chromosome 10, and mutant p53.
- Extracellular matrix metalloproteinase inducer (EMMPRIN) is more frequently expressed in the intestinal-type carcinoma than diffuse-type lesion, and is used as a good marker to differentiate between both kinds of carcinomas. Higher EMMPRIN expression in intestinal-type gastric carcinomas underlies the molecular basis of their high proliferation.

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