Correlation between sialyl Tn antigen and lymphatic metastasis in patients with Borrmann type IV gastric carcinoma

Y Kakeji[1], Y Maehara[2], M Morita[1], A Matsukuma[1], M Furusawa[1], I Takahashi[2], T Kusumoto[2], S Ohno[2] and K Sugimachi[2]

[1]Department of Gastroenterologic Surgery, National Kyushu Cancer Center, and [2]Department of Surgery II, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

Summary The expression of sialyl Tn (STn) antigen in 180 patients with Borrmann type IV gastric carcinomas was examined immunohistochemically. The rate of positive STn staining was 32% (57/180) for the primary tumours, and this positive staining correlated well with tumour extension, lymph node metastasis ($P<0.05$) and peritoneal dissemination ($P<0.01$). One-third (5/15) of patients with positive STn-staining cancer cells had a high level of serum STn. Lesions with positive STn staining were related to a lower survival rate for the patients ($P<0.05$). Proliferative activity of the tumour, as measured by proliferating nuclear antigen (PCNA) labelling percentage and argyrophilic nucleolar organiser region (AgNOR) count, was significantly higher ($41.5 \pm 13.0\%$, 3.78 ± 0.98) in the STn-positive group than in the STn-negative group ($34.2 \pm 13.2\%$, 3.48 ± 0.85) ($P<0.01$, $P<0.05$ respectively). Estimating STn antigen may be useful for predicting the likelihood of lymph node metastasis or peritoneal dissemination and the clinical prognosis for patients with Borrmann type IV gastric carcinoma.

Keywords: sialyl Tn antigen; Borrmann type IV; gastric carcinoma; lymphatic metastasis

Despite advances in diagnostic and surgical management, patients with Borrmann type IV gastric carcinoma have a poor prognosis. Advanced carcinoma of the stomach can be classified, based on Borrmann's criteria, into one of four types (Borrmann, 1926). Borrmann type I carcinoma is a polypoid fungating, type II ulcerative lesion with elevated and distinct borders, and type III is ulcerative but with indistinct borders. Borrmann type IV carcinoma is a diffuse malignant lesion with indistinct borders, and is usually identified only at a very advanced stage (Borchard, 1990; Maehara et al., 1992). The lack of sharp borders of the tumour can make for an underestimation of size. The clinical course is usually unfavourable and the 5 year survival rates are 0–20% (Furukawa et al., 1988; Maehara et al., 1992). A detailed study focusing on the biological behaviour of these highly malignant carcinomas may perhaps improve the prognosis.

Associated with neoplastic transformation, incomplete synthesis of glycolipids or glycoproteins in cell membranes often occurs, resulting in a storage of precursor structures that, in normal cells, would remain cryptic because of further elongation (Hakomori and Kannagi, 1983; Springer, 1984). Sialyl Tn antigen (STn) is one of these abnormal O-linked glycoproteins. The Tn antigen is a precursor of the Thomsen–Freidenreich antigen, the T antigen, and STn is a sialic acid-bound Tn antigen (Hakomori and Kannagi, 1983; Springer, 1984; Kjeldsen et al., 1988). The relationship between STn expression and biological behaviour of cancer cells has been investigated in some human malignancies. In colonic tissues, Itzkowitz et al. (1990) reported a poor outcome for STn antigen-positive patients. Kobayashi et al. (1992) concluded that a positive STn antigen in sera was an independent predictor of a poor prognosis in patients with ovarian cancer. We reported that elevated serum STn levels correlate with advanced tumour stage and a worse prognosis of patients with gastric cancer (Takahashi et al., 1993, 1994). Immunohistochemically, Ma et al. (1993) and Werther et al. (1994) reported that expression of STn antigen may be a useful prognostic marker in patients with gastric cancer. However, the question remained as to whether the lower

survival rate of patients with higher STn expression reflects a higher tumour burden.

To better understand the biological behaviour of STn-positive cancer cells in the most advanced stage, we performed immunostaining for STn antigen in Borrmann type IV gastric cancer and the relationship between STn expression and clinicopathological features was examined with regard to clinical prognosis. We also analysed cell proliferative activity determined by proliferating nuclear antigen (PCNA) labelling percentage and argyrophilic nucleolar organiser region (AgNOR) count, both serving as a parameter of proliferating cells (Egan & Crocker, 1993; McCormick & Hall, 1992).

Materials and methods

Patients

The 180 Japanese patients with primary Borrmann type IV gastric cancer studied herein had undergone gastrectomy in the National Kyushu Cancer Center, Fukuoka, Japan, from 1972 to 1990. A thorough histological examination was made on haematoxylin and eosin-stained preparations, and the histological classification was according to the tumour–node–metastasis classification system of the International Union Against Cancer (UICC, 1987). No patient had been given cytotoxic drugs preoperatively. Post-operative adjuvant chemotherapy was prescribed for 171 patients.

Immunohistochemical staining for STn

Sections from paraffin blocks were dewaxed and stained using the avidin–biotin–peroxidase complex method. The primary antibody TKH2, a monoclonal mouse antibody for sialosyl Tn antigen, was kindly provided by Otsuka Assay Laboratory (Tokushima, Japan). The slides were incubated with fresh 0.3% hydrogen peroxide in methanol for 10 min, then washed three times with phosphate-buffered saline (PBS; pH 7.4). Five per cent normal goat serum in PBS was then applied for 15 min. The sections were incubated overnight with TKH2 (dilution 1:50) at room temperature, with biotinylated goat anti-mouse IgG (1:200 for 30 min; Vector Laboratories) and with the avidin–biotin–peroxidase complex (for 30 min; Vector Laboratories). Peroxidase labelling was developed with 3,3'-diaminobenzidine and hydrogen per-

oxide and the sections were counterstained with haematoxylin.

To ensure the consistency of STn staining between batches, a known positive control gastric carcinoma was included in each round. Negative controls were prepared by substituting normal mouse serum for primary antibody, the results being no detectable staining.

Cellular localisation of the antigenic sites was determined by two investigators without knowledge of clinicopathological information. A double-headed light microscope was used. Scoring was made by examining all low-power optical fields (10 × objective) containing tumour cells and the percentage of antigen-positive cells was estimated. A positive value was recorded if more than 5% of the tumour cells expressed STn antigen.

Serum STn levels

Serum STn levels were measured for 27 patients surgically treated in the period from 1981 to 1986, using a one-step radioimmunoassay kit (S-Tn Otsuka; Otsuka Assay Laboratories, Tokushima, Japan) (Imamura et al., 1989). Competitive binding to the radiolabelled monoclonal antibody TKH2 between serum STn and STn-coated beads was used (an immunoradiometric competitive inhibition assay) (Kjeldsen et al., 1988). Venous blood samples were obtained from patients after an overnight fast and were immediately centrifuged and the serum placed in liquid nitrogen until assay. The cut-off between normal and elevated STn titres was set at 45 U ml^{-1}; this is the mean +two standard deviations (s.d.) of the STn value in normal volunteers, as reported by Imamura et al. (1989).

Proliferative activities

The avidin–biotin–peroxidase complex method was used for PCNA staining, as described elsewhere (Kakeji et al., 1994). The primary antibody was PC10 (Dako, Carpinteria, CA, USA). The PCNA labelling percentage was determined by observing 1000 nuclei in areas of the section with the highest labelling percentage, and the percentage of PCNA-labelled nuclei was used for analysis. For AgNOR staining, the one-step silver colloid method was used. The NOR staining solution was prepared according to the description of Ploton et al. (1982). On the AgNOR-stained slides, careful focusing made visible the AgNOR in the nucleus, in the form of black dots. One hundred cells from each lesion were analysed and a mean score of AgNOR count was recorded.

Statistical analysis

Clinicopathological data were stored in an IBM (Armonk, NY, USA) 4381 mainframe computer. The Biomedical Computer Program (BMDP) was used for all statistical analyses (Dixon, 1988). The BMDP P4F and P3S programs were used for the chi-square test, and the Mann–Whitney test was used to compare characteristics between positive and negative groups with STn staining. The BMDP P1L program was used for analysing survival rates, together with the Kaplan–Meier method, and for testing equality of survival curves, using the method of Mantel and Cox. In the statistical analysis, deaths due to causes other than gastric carcinoma were considered censored cases.

Results

STn staining and clinicopathological characteristics

In the normal gastric mucosa, positive staining for STn was recognised in parietal cells with STn expression in intracellular canalicular membranes. In cancer tissues, the intensity and incidence of staining varied widely from case to case and from area to area within one case. In general, however, the staining was diffusely cytoplasmic, with strong staining associated with the luminal surface (Figure 1). Positive STn staining was evident in 57 (32%) of 180 primary tumours. No obvious relation was found between STn staining and the gender or age of the patient (Table I). All seven patients in whom tumour invasion was confined to the subserosa (T2) showed negative STn staining, and the rate of positive staining increased in proportion to invasion into the deeper layers ($P<0.05$). STn-positive tumours were associated with a higher incidence of metastasis to lymph nodes and peritoneal dissemination than were STn-negative tumours ($P<0.05$, $P<0.01$, respectively).

STn staining and serum STn level

Table II shows the compatibility of histopathological STn staining and serum STn level. Although the patient numbers were too few for a statistical significance, the data do provide interesting information. All 12 with STn-negative cancer cells in tissues had a low level of serum STn, and one-third (5/15) of the patients with STn-positive cancer cells had a high level of serum STn. Table III shows clinicopathological features of patients with immunohistochemically STn-positive cancer cells, according to serum STn levels. In this analysis of 15 cases, there were no obvious features in patients with high STn levels in tissues or in serum. Though not statistically significant, the mean survival time for patients with high serum STn levels (>45 U ml^{-1}) was 298 (131–474) days, that is much shorter than the 778 (148–2932) days for those with low serum STn levels (<45 U ml^{-1}).

STn staining and prognosis

The overall survival curve is shown in Figure 2a, according to positive or negative STn staining. Those with positive STn cancers showed worse survival rates than did those with negative STn cancers ($P<0.05$). Even in 'curative' cases, there was a tendency toward a shorter survival for those with positive STn cancers (Figure 2b). Table IV shows the pattern of recurrence, based on STn staining. Though the recurrence rate tended to be higher in patients with STn-positive tumours (13/18; 72%) than that in those with STn-negative tumours (38/65; 58%), no obvious difference was recognised in the pattern of recurrences.

STn staining and proliferative activity

The proliferative activity expressed by mean PCNA labelling percentage was 41.5% for STn-positive cases, a value significantly higher than 34.2% for the STn-negative cases ($P<0.01$, Table V). The mean AgNOR count for STn-positive cases (3.77) was also significantly higher than that for STn-negative cases (3.48, $P<0.05$).
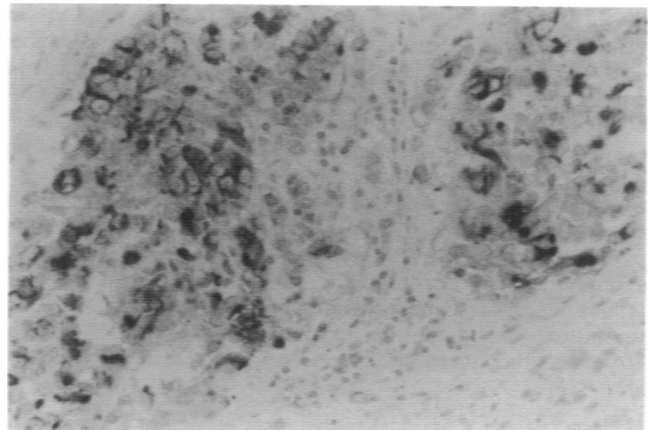

Figure 1 Gastric carcinoma of moderately differentiated type stained with TKH-2 antibody and showing both cytoplasmic and apical reactivity (× 200).

Table I Clinicopathological characteristics and STn staining

Histological findings	STn staining Negative (n = 123)	STn staining Positive (n = 57)	P-value
Sex			
Men	70	28	NS
Women	53	29	
Mean age (years)	55.8±11.7	58.6±14.0	NS
Tumour size (cm)	12.2±4.0	13.2±4.0	NS
Histological type			
Well differentiated	1	2	NS
Moderately differentiated	11	9	
Poorly differentiated	51	13	
Signet	59	26	
Mucinous	1	7	
Tumour extension			
pT2	7	0	P<0.05
pT3	84	31	
pT4	32	26	
Invasion into lymphatics			
No invasion	8	1	NS
Slight invasion	49	21	
Moderate invasion	40	14	
Severe invasion	26	21	
Venous invasion			
No invasion	43	15	NS
Slight invasion	73	36	
Moderate invasion	6	5	
Severe invasion	1	1	
Lymph node involvement			
pN0	17	4	P<0.05
pN1	28	8	
pN2	59	26	
pM1	19	19	
Peritoneal dissemination			
Negative	98	31	P<0.01
Positive	25	26	
Metastasis to the liver			
Negative	119	56	NS
Positive	4	1	
Stage			
IA	0	0	P<0.01
IB	1	0	
II	13	0	
IIIA	20	4	
IIIB	32	10	
IV	57	43	
Curability			
Curative	66	17	P<0.01
Non-curative	57	40	

Table II Compatibility of histopathological STn staining and serum STn level

Serum STn level (U ml⁻¹)	STn staining Negative	STn staining Positive
<45	12	10
>45	0	5

Table III Clinicopathological characteristics of patients with immunohistochemically STn-positive cancer cells according to serum STn level

Histological findings	Serum STn level (U ml⁻¹) <45 (n = 10)	Serum STn level (U ml⁻¹) >45 (n = 5)
Sex		
Men	5	4
Women	5	1
Mean age (years)	50.1±11.1	72.8±7.6*
Tumour size (cm)	12.9±3.8	14.3±2.4
Histological type		
Well differentiated	1	0
Moderately differentiated	3	1
Poorly differentiated	2	0
Signet	4	2
Mucinous	0	2
Tumour extension		
pT2	0	0
pT3	5	3
pT4	5	2
Invasion into lymphatics		
No invasion	0	0
Slight invasion	4	1
Moderate invasion	3	1
Severe invasion	3	3
Venous invasion		
No invasion	3	1
Slight invasion	7	4
Moderate invasion	0	0
Severe invasion	0	0
Lymph node involvement		
pN0	1	0
pN1	1	0
pN2	4	2
pM1	4	3
Peritoneal dissemination		
Negative	5	4
Positive	5	1
Metastasis to the liver		
Negative	9	4
Positive	1	1
Stage		
IIIB	1	1
IV	9	4
Curability		
Curative	3	2
Non-curative	7	3
Mean survival (days)	778 (148–2932)	298 (131–474)

*P<0.01.

Discussion

Quantitative differences in cell-surface sialic acid (total cell-surface sialic acid levels) and qualitative changes in sialylated oligosaccharides (presence or absence of a particular oligo-saccharide structure or individual glycoconjugates) are commonly associated with metastasis (Yogeeswaran and Salk, 1981; Altevogt et al., 1983; Passaniti and Hart, 1988). The finding that STn-positive cancers in primary lesions correlated with lymph node metastasis and with peritoneal metastasis suggests that the STn antigen may be drained specifically by the lymphatic route. The specific drainage route for tumour cells with special antigen was discussed by Tabuchi et al. (1990); they stated that CA 19-9, a cancer-associated antigen with sialylated carbohydrates, may be drained primarily by the thoracic duct of the lymphatic system via node metastases or invasive lymphatics. Furthermore, tumours with positive STn staining had a higher proliferative activity than did those with which stained negatively. We have previously reported that tumours with a high proliferative activity often metastasise to lymph nodes (Kakeji et al., 1991, 1994). Therefore, this high proliferative activity will probably accelerate the lymphatic spread of cancer cells through association with altered cell membrane glycoproteins. As patients with Borrmann type IV carcinoma are more likely to have lymph node metastases and peritoneal dissemination than those with other types (Maehara et al., 1992), STn may be an additional predictor of survival time for those patients.

Carbohydrate antigens with changes in glycosylation, detected in patients' serum, have been used as tumour
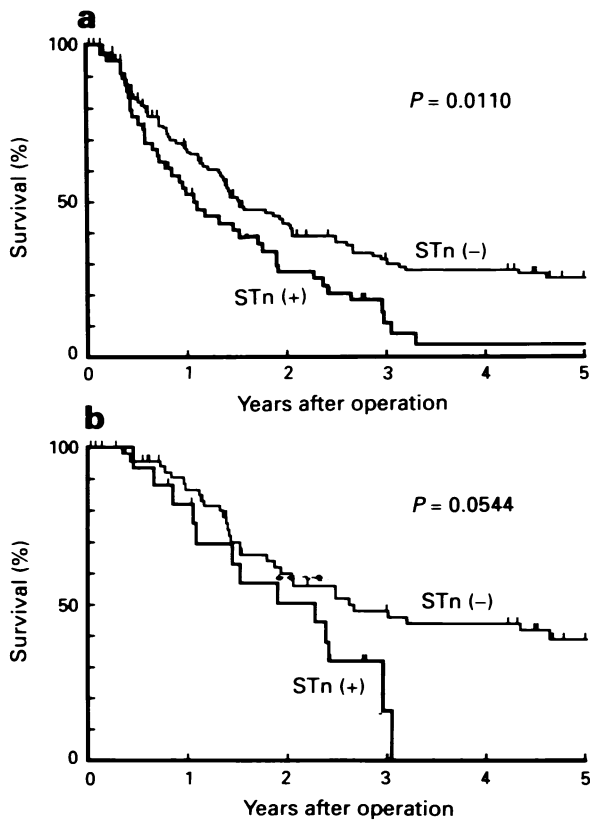
Figure 2 The survival curves of patients with Borrmann type 4 gastric carcinoma. The thin black line indicates cases with STn-negative tumour and the bold black line indicates those with STn-positive tumour. a, Survival curves for all patients. There was a significant difference ($P<0.05$). b, Survival curves of 'curative' cases.

Table IV STn staining and pattern of recurrence

	STn staining	
	Negative	Positive
Pattern of recurrence	(n = 65)	(n = 18)
Peritoneal dissemination	23	5
Metastasis to lymph nodes	4	5
Liver metastasis	4	0
Pulmonary metastasis	2	0
Bony metastasis	0	1
Local recurrence	2	2
Other recurrences	3	0
Total recurrences	38	13
No recurrence	27	5

Table V STn staining and proliferative activity

	STn staining (mean ± s.d.)	
	Negative	Positive
Proliferative activity	(n = 123)	(n = 57)
PCNA labelling (%)	34.2 ± 13.2	41.5 ± 13.0**
AgNOR count	3.48 ± 0.85	3.78 ± 0.98*

*$P<0.05$, **$P<0.01$.

markers. As for STn antigen, it is not well known whether the levels of circulating serum STn antigen reflect changes in expression of this antigen on cancer cell membranes. The positive rates of STn antigen in immunohistochemical and serological study have been noted for some human cancers. In patients with colorectal cancer, the positive rate in tissue was 88% (112/128) (Itzkowitz et al., 1990) and that in serum was 28% (5/18) (Motoo et al., 1991). Those rates in patients with endometrial cancers were 84% (36/43) in tissue (Inoue et al., 1991) and 5% (2/42) in serum (Inoue et al., 1990). We found little documentation of comparisons of the STn levels

in tissues and those in sera from the same patients. As for data on gastric cancer, together with data from our previous report (Takahashi et al., 1993), we noted a relationship between the expression of STn by immunohistochemical staining and the serum STn antigen level. In about one-third of the cases of gastric cancer, cell-surface STn glycoconjugate seems to be drained into the systemic circulation. No particular characteristic emerged from cases of ready shedding of STn antigen into vessels. Patients with elevated serum STn antigen levels survived for only a short time, perhaps because of the higher tumour burden.

Even among patients with the same Borrmann type IV carcinoma, there are variations in lifespan. STn is associated with lymph node metastasis and peritoneal dissemination in this lesion, therefore this antigen may be one predictor of survival time for patients with Borrmann type IV carcinoma.

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