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Expression of Globo-series Gangliosides in Human Renal Cell Carcinoma

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Gangliosides have been shown to be involved in development, differentiation, oncogenesis, and cancer progression. We investigated immunohistochemical expression of globo-series gangliosides in human renal cell carcinoma (RCC) and whether their expression is related to the clinical course. The expression of globo-series gangliosides was evaluated in fresh-frozen sections of 55 primary renal tumors and 8 metastatic deposits using monoclonal antibodies RM1 and RM2, which define monosialosyl and disialosyl galactosylgloboside, respectively. The immunoreactivity of primary tumors to RM1 and/or RM2 was correlated with the clinicopathological data. Cumulative incidence of metastasis detected at initial diagnosis and during the follow-up period was significantly higher in the cases whose primary tumors were RM1/RM2-positive (RM1 and/or RM2-positive) than in the RM1/RM2-negative (neither RM1 nor RM2-positive) cases ($P < 0.05$). During the follow-up period, metastasis developed in none of the RM1/RM2-negative cases which had not shown metastasis at initial diagnosis. High nuclear grade was observed only in the RM1/RM2-positive cases. The RM1/RM2-positive rate of the metastatic deposits was higher than that of the primary tumors. Furthermore, a metastatic deposit obtained from one of the cases whose primary tumors were equivocal for RM1/RM2 was extensively stained by RM1 and RM2. These results indicate that globo-series gangliosides may be one of the biochemical indicators related to the metastatic potential of human RCC.

Key words: Glycolipid — Globo-series ganglioside — Human renal cell carcinoma — Metastatic potential

Human renal cell carcinoma (RCC) is highly metastatic. About 30% of patients already have metastasis at the time of initial diagnosis, and metastasis develops in 30–50% of patients who undergo radical operation.[1–3] In other words, detectable or undetectable metastases have already developed in 60–80% of patients at the time of initial diagnosis of RCC. However, there has been no reliable indicator of biological malignancy in regard to metastatic potential, and no effective therapy for metastasis, except surgery and immunotherapy.

Gangliosides are membrane-bound glycolipids containing sialic acids, and glycolipids are classified into globo-, lacto-, and ganglio-series based on the core structure of the sugar chain. The carbohydrate moieties of gangliosides undergo profound changes during development, differentiation, and oncogenesis,[4] and altered glycosylation of glycolipids and glycoproteins is observed in cancer progression, although the mechanism remains unknown.[5] In colorectal cancer, sialosyl-Tn expression has prognostic implications,[6] and sialosyl-Le^x expression is correlated with metastatic potential[7] and poor survival.[8] In lung cancer, expression of H/Le^y/Le^b antigens is inversely correlated with survival.[9]

In our previous studies on glycolipids of human RCC, we found that slower-migrating gangliosides increase in metastatic deposits,[10] globo-series gangliosides are the major components of the slower-migrating gangliosides (Fig. 1),[11] and disialosyl galactosyl globoside (DSGG) is an adhesion molecule.[12] To investigate further whether expression of globo-series gangliosides is associated with biological malignancy of human RCC, we performed immunohistochemical staining of fresh-frozen human RCC sections using monoclonal antibodies (mAbs) RM1 and RM2,[11] which define monosialosyl galactosyl globoside (MSGG) and DSGG, respectively. To our knowledge, this is the first demonstration of globo-series gangliosides on human RCC by immunohistochemistry.

MATERIALS AND METHODS

Tissues Primary tumor tissues of human RCC were obtained from 55 patients who had undergone nephrectomy in Tohoku University Hospital, Japan, and its associated hospitals from September 1990 to February 1996. Fifty-one patients had transperitoneal radical nephrectomy, and 4 had lumbar nephrectomy. Metastatic deposits were available from 8 patients, who had back subcutaneous, lymphnode (3 cases), lung, adrenal, pancreas, and bone metastasis, respectively. Immediately after resection, tissues were embedded in OCT compound, frozen in liquid nitrogen and stored at −80°C until use. Histological stage was determined according to the TNM classification of malignant tumors (UICC, 1992). Nuclear grading was judged on formalin-fixed paraffin-embedded sections stained with hematoxylin and eosin, based on the degree of nuclear atypia according to

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the General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma (Japanese Urological Association, 1992). Metastasis was evaluated by chest roentgenography, computed tomography, and bone scintigraphy.

Antibodies mAbs RM1 and RM2, directed to MSGG and DSGG, respectively, were established using RCC cell line TOS1, derived from a metastatic deposit, as described previously.[11]

Immunohistochemical staining of tissue sections by RM1 and RM2 Avidin-biotin immunoperoxidase staining was performed using a Vector staining kit (Burlingame, CA). Frozen tissues were cut (5 μm thickness), air-dried, and fixed in acetone for 2 min. Endogenous peroxidase was blocked with 0.03% H_2O_2 in phosphate-buffered saline. Sections were blocked with normal goat serum for 30 min. After blocking of endogenous biotin using a kit from Vector, sections were incubated with primary antibody for 30 min, incubated with biotinylated secondary antibody for 30 min, incubated with Vector avidin-biotin solution for 30 min, and stained with 3′,3′-diaminobenzidine substrate solution containing 0.03% H_2O_2. Mouse IgM was used as a negative control.

Evaluation of immunohistochemistry and statistical analysis *Immunoreactivity was graded as follows:* +++, more than 50% of tumor cells were positive; ++, 10–50% were positive; +, less than 10% were positive; −, no positive tumor cells were found. Sections showing − were considered negative, and sections showing ++ and +++ were considered positive. Sections showing + were considered equivocal. The association between the variables was statistically assessed excluding the equivocal cases.

RESULTS

In normal kidney, distal tubules and the loop of Henle were positively stained, but proximal tubules and glomer-

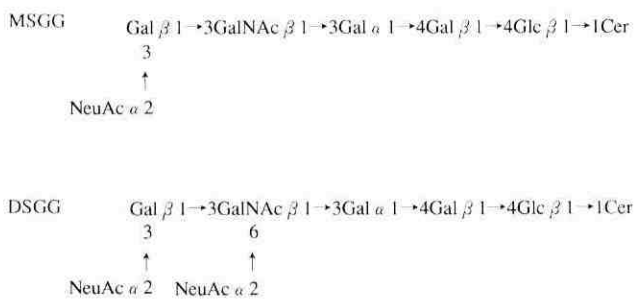

Fig. 1. Structures of globo-series gangliosides. Monoclonal antibody (mAb) specific to MSGG is RM1, and mAb specific to DSGG is RM2. MSGG, monosialosyl galactosyl globoside; DSGG, disialosyl galactosyl globoside; Cer, ceramide.

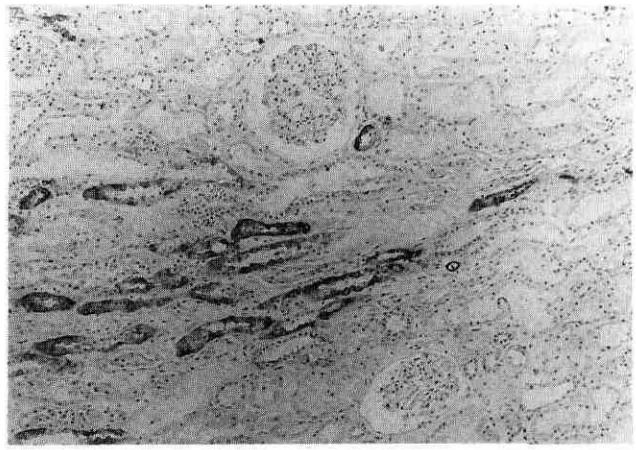

Fig. 2. Normal kidney section stained by RM2. Distal tubules are positively stained, but proximal tubules and glomeruli are not stained (×100).

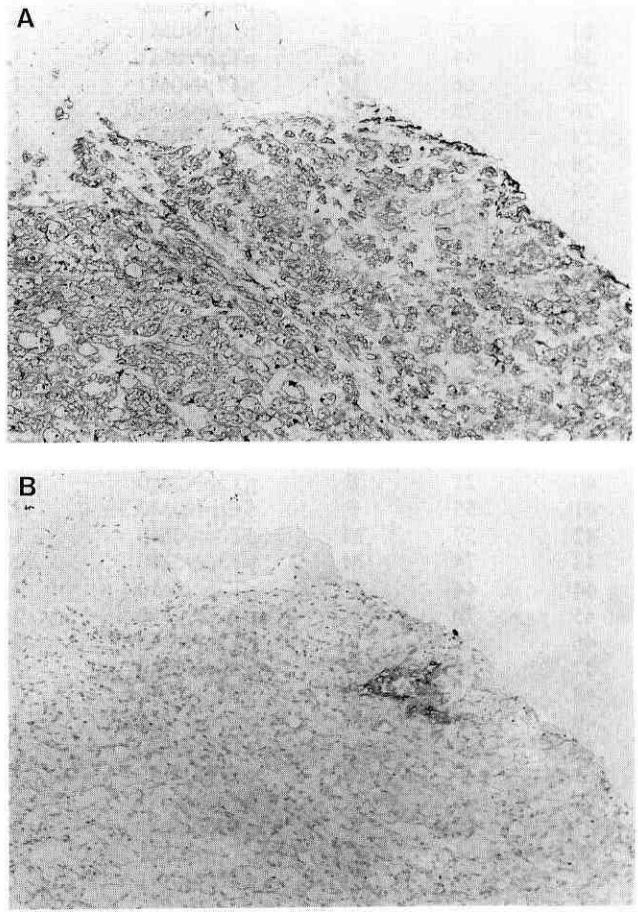

Fig. 3. Immunostaining of primary tumor sections (case 14). A, staining by RM1 (×100); B, staining by RM2 (×100).

Table I. Clinicopathological Data of Renal Cell Carcinoma

Case	Age	Sex	Stage	Grade	Cell type	Tumor size (cm)	RM1	RM2
1	58	M	pT2pN1M0	2	clear	9	+++	+++
2	59	M	pT2pN0M0	2	clear	9.5	+++	+++
3	69	M	pT2pN0M0	2	clear	7.5	+++	+++
4	56	M	pT2pN2M0	2	granular	4.5	+++	++
5	69	M	pT2pN0M1	2	clear	7	+++	++
6	62	M	pT3apN0M1	2	clear	5	+++	++
7	66	M	pT2pN0M0	2	clear	5	+++	++
8	61	M	pT2pN0M0	2	clear	4	+++	++
9	71	M	pT2pN0M0	3	clear	4.5	+++	++
10	59	F	pT2N0M0	2	clear	3	+++	++
11	33	M	pT2pN0M0	2	clear	6.5	+++	++
12	55	M	pT3pN0M1	2	clear	6	+++	++
13	66	M	pT2pN2M0	2	mixed	5.5	+++	+
14	62	M	pT2pN0M0	1	clear	4	+++	+
15	61	M	pT3pN0M1	2	clear	6	+++	−
16	71	M	pT3bpN0M1	2	clear	6.5	+++	−
17	41	M	pT3bpN0M1	3	clear	9	+++	−
18	72	M	pT2pN0M0	1	clear	3	+++	−
19	70	M	pT1pN0M0	2	clear	2	+++	−
20	72	M	pT2pN0M0	2	clear	8	+++	−
21	46	M	pT2pN0M0	2	mixed	3.5	+++	−
22	61	M	pT1pN0M0	1	clear	2.5	+++	−
23	62	M	pT2pN0M1	2	clear	5	++	++
24	54	M	pT2pN0M1	3	mixed	5	++	++
25	66	M	pT2pN0M1	1	clear	5.5	++	++
26	75	M	pT3apN0M0	2	granular	2.5	++	++
27	63	F	pT3pN0M0	2	clear	5	++	+
28	59	M	pT3bpN1M0	2	mixed	9	++	−
29	74	M	pT3apN0M1	2	clear	9.5	++	−
30	70	F	pT3pN0M0	3	clear	13.5	++	−
31	76	M	pT2pN0M0	2	clear	6	++	−
32	71	M	pT3bpN0M0	1	clear	5.5	++	−
33	63	F	pT3bpN0M0	2	clear	10	−	+++
34	70	M	pT2pN0M1	3	mixed	10	−	++
35	51	M	pT2pN0M1	2	clear	4	+	−
36	60	F	pT3pN0M1	2	clear	14	+	−
37	62	M	pT3pN0M0	2	clear	4	+	−
38	71	M	pT2pN0M0	2	clear	3	+	−
39	59	M	pT2N0M0	1	clear	3	+	−
40	21	F	pT2pN2M0	2	clear	6	+	−
41	55	F	pT2pN0M0	2	clear	6	−	−
42	59	M	pT2pN0M0	1	clear	10	−	−
43	74	M	pT3pN0M0	2	granular	5	−	−
44	44	F	pT2pN0M0	2	clear	5	−	−
45	70	F	pT2pN0M0	2	clear	9	−	−
46	52	F	pT2pN0M0	2	granular	10	−	−
47	59	F	pT2pN0M0	1	clear	3	−	−
48	71	M	pT2pN0M0	1	clear	12	−	−
49	75	M	pT3bpN0M0	2	clear	9.5	−	−
50	51	M	pT2pN0M0	1	clear	4	−	−
51	39	M	pT2N0M0	2	clear	5	−	−
52	76	F	pT3apN0M0	1	clear	4.5	−	−
53	49	F	pT3pN0M1	2	granular	9.5	−	−
54	66	F	pT4pN0M1	2	clear	9	−	−
55	22	M	pT2pN0M1	2	granular	5.5	−	−

AVQ, doxorubicin+vincristine+carboquone; IFN, interferon; LN, lymphnode; meta, metastasis; mos, months; NED, no evidence of disease; TAE, transarterial embolization.

Metastasis at diagnosis	Metastasis during follow-up (mos)	Adjuvant therapy (mos)	Outcome (mos)
para-aortic LN		IFNα (1)→none	NED (51)
		none	NED (26)
	lung (30), bone (34)	none→IFNα	alive (35)
para-aortic LN	lt. neck LN (3)	IFNα	died (12)
lung		IFNα	died (5)
adrenal gland, bone	brain (5), lung & liver (6)	IFNα, chemoradiation to bone meta.	died (9)
		none	NED (18)
	lung & gallbladder (36)	none→IFNα, tegafur, uracil p.o.	alive (56)
		none	NED (26)
		AVQ 1 course, IFNα (2)→none	NED (39)
		none	NED (21)
lung		IFNα	died (12)
para-aortic LN	bone (11)	IFNα, chemoradiation to bone meta.	alive (43)
		none	NED (38)
lung		IFNα, tegafur, uracil p.o.	alive (41)
lung	brain (5)	IFNα, γ-knife for brain meta.	died (10)
lung		IFNα, TAE for lung meta.	died (31)
	lung (21)	none	died (28)
		carboquone p.o. (24)→none	NED (67)
		none	NED (19)
		none	NED (18)
		none	NED (14)
back, brain		extirpation of back and brain meta.	died (2)
bone		IFNα	died (5)
bone		IFNα, extirpation of bone meta.	died (12)
		none	NED (16)
		none	NED (15)
para-aortic LN	lung (1)	none→IFNα for lung meta.	alive (29)
brain		γ-knife for brain meta.	NED (25)
	lung (15), liver (39)	none→IFNα, TAE for liver meta.	died (54)
		none	NED (39)
		IFNα	NED (28)
		IFNα (24)→tegafur, uracil p.o.	NED (52)
lung	bone (3)	IFNα	died (12)
bone		IFNα	died (30)
lung, brain		IFNα, γ-knife for brain meta.	died (19)
	lt. neck LN (37)	none→resection of LN meta., IFNα	alive (41)
		tegafur, uracil p.o. (12)→none	NED (38)
		tegafur, uracil p.o. (2)→none	NED (24)
para-aortic LN		IFNα	NED (18)
		none	NED (54)
		none	NED (69)
		none	NED (54)
		none	NED (51)
		tegafur, uracil p.o.	NED (53)
		none	NED (34)
		none	NED (31)
		none	NED (33)
		IFNα (1)→none	NED (40)
		none	NED (20)
		AVQ 1 course, IFNγ (1)→none	NED (40)
		none	NED (24)
lung	liver (1)	IFNα	died (4)
pancreas, lung		tegafur, uracil p.o.	alive (13)
bone		IFNα, chemoradiation to bone meta.	alive (15)

uli were not stained by RM2 (Fig. 2). RM1 gave the same staining pattern as RM2, but the staining area by RM1 was smaller than that by RM2.

Thirty-two (58%) primary tumors were positively stained by RM1, and 18 (33%) were positively stained by RM2. Thirty-four out of the 55 primary tumors were positively stained by RM1 and/or RM2 (Fig. 3, and Table I), and 15 of these 34 RM1/RM2-positive cases showed metastasis at the time of initial diagnosis. Fifteen primary tumors were stained by neither RM1 nor RM2. Three of these 15 RM1/RM2-negative cases showed metastasis at the time of initial diagnosis. After operation, 7 patients without metastasis at initial diagnosis received adjuvant therapy, and these cases were excluded from the follow-up study. Among the cases which did not show metastasis at the time of initial diagnosis, four of the remaining 15 RM1/RM2-positive cases showed metastasis (mean follow-up until development of metastasis; 23.5 months), whereas none of the remaining 9 RM1/RM2-negative cases showed metastasis (mean follow-up; 41.2 months) during the follow-up period ($P<0.1$). Thus, excluding 6 RM1/RM2-equivocal cases, the cumulative incidence of metastasis was 19 of 30 RM1/RM2-positive cases versus 3 of 12 RM1/RM2-negative cases

Table II. Correlation of Expression of Globo-series Gangliosides in Primary Renal Tumor and Incidence of Metastasis

	Incidence of metastasis		Cumulative incidence of metastasis	
	at diagnosis	during follow-up		
RM1/RM2-positive	15/34	4/15	19/30	$P<0.05$
RM1/RM2-negative	3/15	0/9	3/12	

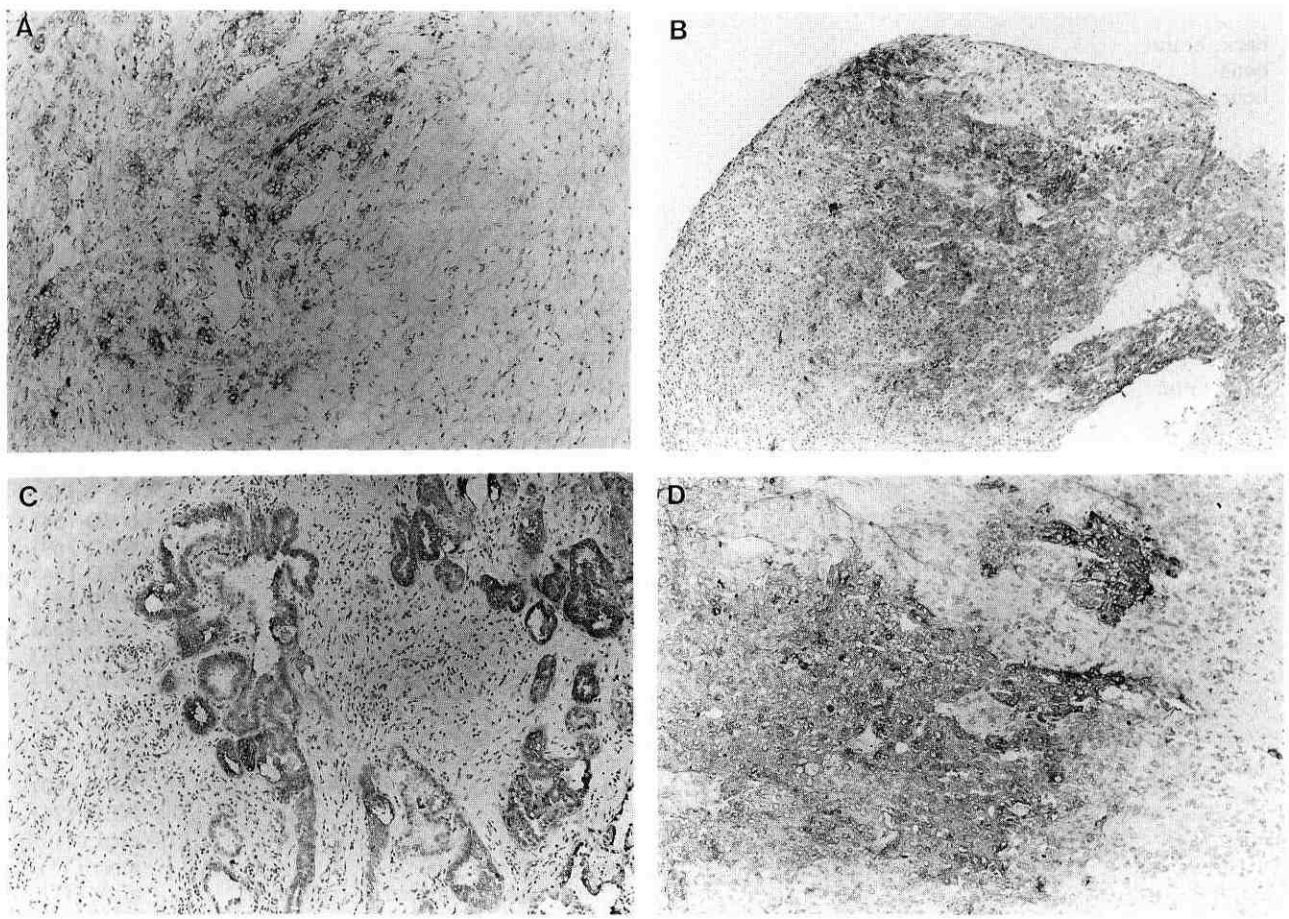

Fig. 4. Immunostaining of metastatic deposit sections. A, back subcutaneous metastasis (case 23) stained by RM2. Tumor cells stained by RM2 are surrounded by subcutaneous adipose tissue (×100). B, adrenal gland metastasis (case 6) stained by RM1(×100). C, lymphnode metastasis (case 13) stained by RM1 (×100). D, lung metastasis stained by RM1 (×100).

Table III. Immunoreactivity of Metastatic Deposits

Site of metastasis	Case No.	RM1	RM2	Prior therapy
back subcutaneous	23	+++	+++	none
lt. neck LN	37	+++	+++	none
adrenal gland	6	+++	++	none
para-aortic LN	4	+++	++	*none*
para-aortic LN	13	+++	−	none
lung	/	++	−	IFNα
pancreas	54	−	−	none
bone	55	−	−	none

($P<0.05$) (Tables I and II). Six of the eight (75%) metastatic deposits were positively stained by RM1; four of these (50%) were also stained by RM2 (Fig. 4 and Table III), and the rate of positive staining by RM1/RM2 of the metastatic deposits was higher than that of the primary tumors. Furthermore, the metastatic deposit detected three years after operation, obtained from one of the cases whose primary tumors were equivocal for RM1/RM2, was extensively stained by RM1 and RM2 (Fig. 5). The nuclear grade (grade 3) of this metastatic tumor cells was also higher than that (grade 2) of the primary tumor cells. High nuclear grade (grade 3) was observed in 5 out of 34 RM1/RM2-positive cases *versus* in none out of 15 RM1/RM2-negative cases (Table I). Interestingly, the RM1/RM2-positive rate of male cases was significantly higher than that of female cases ($P<0.001$). Expression of globo-series gangliosides was not significantly correlated with local invasion (by χ^2 test) or tumor size (by t test). Mean tumor size (6.1 cm) of the RM1/RM2-positive cases was smaller than that (7.1 cm) of the RM1/RM2-negative cases.

DISCUSSION

This report shows that expression of globo-series gangliosides in human RCC is significantly correlated with the development of metastasis. The findings of this report, along with our previous study[12] showing that globo-series gangliosides were expressed in RCC cell lines derived from metastatic deposits, indicate that globo-series gangliosides may reflect the metastatic potential of human RCC. The case whose metastatic deposit was extensively stained by RM1/RM2, in striking contrast to the low-grade staining of the primary tumor, may be a typical example. This case is also consistent with the finding that specialized subpopulations of cells overcome the highly selective host defense mechanism to form metastasis.[13]

During the follow-up period, metastasis developed in none of the RM1/RM2-negative cases which had not shown metastasis at initial diagnosis. From the clinical

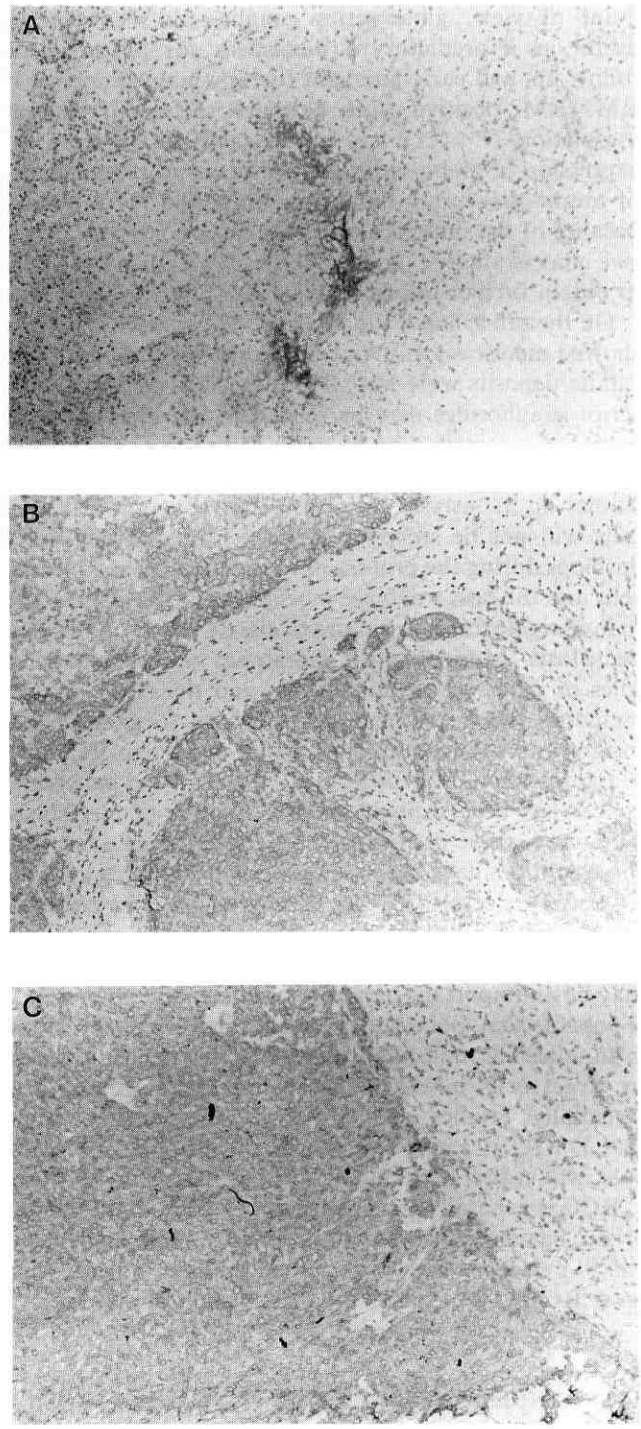

Fig. 5. Immunostaining of primary and metastatic tumors from the same case (case 37). A, primary tumor section stained by RM1 (×100). Only a few tumor cells are stained and the immunoreactivity is classified as equivocal. This tumor section is not stained by RM2. B and C, metastatic tumor sections stained by RM1 (×100) and RM2 (×100), respectively. Most tumor cells are stained in striking contrast to the low grade staining of the primary tumor.

point of view, globo-series gangliosides may become useful as a predictor of metastasis for postoperative follow-up, and early adjuvant therapy may be applied to RM1/RM2-positive cases, although so far only a weak association ($P<0.1$) has been established between the expression of globo-series gangliosides and the incidence of metastasis during the follow-up period. This may be because of the limited number of cases analyzed. Extensive clinical studies and long-term follow-up are required to obtain further information.

On the other hand, the RM1/RM2-negative cases also showed metastasis at initial diagnosis. Two of eight metastatic deposits were not stained by RM1/RM2. Globo-series gangliosides may be related to the metastatic potential of a certain type of RCC. Other factors may also play important roles in the progression of RCC, and if so, studies on metastasis only from the viewpoint of tumor cells will not be sufficient. Considering that metastasis develops during the process of interaction between tumor cells and the host's defense mechanism,[13, 14] we have also to investigate the host factors to clarify the mechanism of metastasis. More detailed researches on the biological roles of globo-series gangliosides and the other factors, including the host ones, are necessary to gain further insight into the metastatic potential of human RCC.

Expression of SLe^x or SLe^a in colorectal and pancreatic cancer has been claimed to be predictive of metastasis. Tumors in these organs often express SLe^x or SLe^a, and these epitopes have been identified as the binding sites for E- and P-selectins.[15–19] In kidney tumors, in contrast, SLe^x expression has no correlation with metastatic potential or tumor progression,[20] and globo-series ganglioside may be an important indicator of metastatic potential. It is possible that globo-series ganglioside in kidney tumors is an adhesion molecule resulting in tumor cell adhesion to target cells in tissues where metastasis occurs preferentially. Studies along this line have recently shown that DSGG is a non-selectin-dependent adhesion molecule, which preferentially adheres to lung.[12]

RCC is generally considered to be of proximal tubular origin, based on ultrastructural[21] and immunohistochemical[22] evidence. However, other studies indicate that most RCCs also express the distal tubular antigens,[23, 24] suggesting divergent histogenesis from a precursor stem cell.[24] Immunostaining of primary kidney tumor tissue reveals the presence of cells both negative and positive for globo-series ganglioside. In normal kidney, globo-series ganglioside is detectable in distal tubular epithelia and the loop of Henle. Therefore, this study supports the latter hypothesis. Further extensive studies on differentiation-dependent changes of globo-series ganglioside may shed light on the histogenesis of RCC.

Extended globo-series structures, particularly MSGG were originally described as stage-specific embryonic antigen 4.[25, 26] MSGG is highly expressed in human embryonal carcinoma cells[27] and presumably in preimplantation human embryo. Synthesis of extended globo-series structures by embryonal carcinoma cells declines upon differentiation, and shifts to synthesis of lacto-series and eventually of ganglio-series structures.[28] Therefore, it is assumed that expression of globo-series gangliosides in RCCs represents a more undifferentiated phenotype than that of tumor cells not expressing globo-series gangliosides.

The expression rate of MSGG was higher than that of DSGG in both primary and metastatic RCC, suggesting that MSGG may be the primary product in the biosynthetic pathway of globo-series gangliosides in human RCC, and the route from MSGG to DSGG may be secondary. It is noteworthy that the expression rate of globo-series gangliosides was significantly higher in male cases. Because of the higher incidence of RCC in men, it would be interesting to investigate whether these facts are associated with each other. We have recently found another type of DSGG (DSGG anomer) in human RCC (Ito, Saito, *et al.*, unpublished results). Researches on globo-series gangliosides may lead to an understanding of the malignant potential of human RCC, and these gangliosides may become useful in the future as new markers for human RCC.

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