High Incidence of GalNAc Disialosyl Lactotetraosylceramide in Metastatic Renal Cell Carcinoma

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Abstract. *Background: In renal cell carcinoma (RCC), glycosphingolipids monosialosyl globopentaosylceramide (MSGb5) and GalNAc disialosyl lactotetraosylceramide (GalNAcDSLc4) were shown to be predictors of metastasis. Here we extended the research using a larger cohort of patients with a longer follow-up period, and reevaluate their relationship to malignant potential, metastasis and prognosis in patients with RCC. Patients and Methods: MSGb5 and GalNAcDSLc4 were examined in 114 primary RCCs by immunohistochemical method on cryostat sections. Results: GalNAcDSLc4 was detected in 13.2% of RCCs and was associated with a significantly higher incidence of metastasis at the time of primary visit (60.0% vs. 31.3%, p=0.0419) as well as de novo metastasis during follow-up (33.3% vs. 14.7%), and a shorter survival (p=0.0399). MSGb5 was detected in 51.8% of tumors and was not related to clinocopathological characteristics or survival. Conclusion: RCC patients with tumors positive for GalNAcDSLc4 are at higher risk of metastasis at the time of diagnosis and during follow-up.*

Approximately one-third of patients with renal cell carcinoma (RCC) have metastatic disease at initial presentation, and 20-30% of patients with localized tumors relapse after radical nephrectomy. The median time before relapse after nephrectomy is 15 to 18 months, and 85% of relapses occur within 3 years (1, 2). However, with some patients manifesting recurrences beyond 5 years of follow-up, continuing surveillance beyond 5 years is necessary (3). Despite definitive therapy, a subset of patients with localized tumors ultimately develop metastatic disease. Although the tumor grade and stage may provide some information regarding the clinical course, it remains unknown which patients are at direct risk of tumor spread or progression. A number of molecular markers of RCC have been tested; however, to date, none has been validated as a better prognostic factor than stage and grade (4, 5).

Gangliosides are glycosphingolipids containing sialic acids and present in the outer leaflet of plasma membranes. Glycosphingolipids are involved in modulating functional membrane proteins, regulating transmembrane signaling and mediating cellular interactions (6). Altered glycosylation of glycosphingolipids is observed in various types of cancer (7). Some studies have reported that aberrant glycosylation expressed in specific types of human cancer (colorectal cancer, lung cancer) may define the stage, and fate, of cancer progression (8-11). In RCC, the correlation between the ganglioside profile and metastatic properties has been studied. The increased expression of ganglioside that migrates slowly on thin-layer chromatography is correlated with metastasis in deposits of RCC (12, 13). The major components of this ganglioside are Globo-series gangliosides. Monosialosyl globopentaosylceramide (MSGb5) and disialosyl globopentaosylceramide (DSGb5) were previously identified in RCC tissue extract (14). Two additional gangliosides, disialosyl lactotetraosylceramide (DSLc4) and GalNAc disialosyl lactotetraosylceramide (GalNAcDSLc4), were shown to be expressed in RCC (15). The expression of MSGb5 and GalNAcDSLc4, defined by monoclonal antibodies (mAbs) RM1 and RM2, respectively, was shown to be correlated with a higher incidence of metastasis in human RCC in a previous pilot study by a collaborative group (16).

In the study reported here, we reinvestigated the expression of MSGb5 and GalNAcDSLc4 immunohistochemically in a larger cohort of patients with a longer follow-up period and evaluated the relationship of these gangliosides to malignant potential, metastasis and prognosis in patients with RCC.

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Patients and Methods

Patients. A total of 114 unselected patients (83 men and 31 women) who underwent surgery for RCC between 1990 and 2001 at the Department of Urology Niigata University Hospital were studied. Their mean age was 58.7 years (range 35 to 87). The median follow-up period was 53.5 months (range 0 to 184). The main clinical and pathological characteristics of the patients are listed in Table I. The histological diagnosis, grading and staging were determined according to the UICC TNM classification (17).

Antibodies. MAbs RM1 and RM2, directed at MSGb5 and GalNAcDSLc4, respectively, were established using the RCC cell line TOS1 isolated from lung metastatic deposits as described elsewhere (14, 15).

Immunohistochemistry. Avidin-biotin immunoperoxidase staining of fresh-frozen tissue sections was performed as described elsewhere (18). Briefly, resected tissues were immediately embedded in OCT compound, frozen in liquid nitrogen and stored at –80˚C. Cryostat sections (5 μm) were placed on poly-L-lysine-coated slides, air-dried and fixed in cold acetone for 10 min. The sections were incubated for 15 min in 20% normal sheep serum at room temperature to block nonspecific binding, followed by avidin and biotin for 10 min each. The sections were then incubated with primary antibody for 1h at room temperature. Incubation was followed by sequential 15-min incubations with biotinylated sheep anti-mouse immunoglobulin (Amersham, Buckinghamshire, UK) and streptavidin-horseradish peroxidase (HRP) (Amersham). The sections were stained with 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in 0.05 M tris-HCl. They were finally counterstained with Meyer's hematoxylin solution (Wako, Japan) and mounted with Mount-Quick (Daido Sangyo Co., Ltd, Japan).

Evaluation of immunostaining. The staining results were evaluated by two independent observers (T. Itoi and R. Maruyama), who had no prior knowledge of the clinical or pathological data. Immunoreactivity was graded according to the criteria used in the pilot study as follows: strong, more than 50% of tumor cells positive; moderate, 10-50%; weak, less than 10%; negative, no positive tumor cells were found. Tumor specimens showing weak staining were considered negative. Those with moderate and strong staining were considered positive.

Statistical analysis. Statistical analysis was performed using StatView 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA). Chi-square and Fisher's exact tests were used to assess the association between the expression and clinicopathologic parameters. The Kaplan-Meier method was used to determine survival, and the log-rank test was used to compare survival curves. All *p*-values less than 0.05 were considered statistically significant.

Table I. *Patient characteristics.*

Mean follow-up period (range) in months	61.4 (0-184)
Mean age (range) in years	58.7 (35-87)
	Number of patients
Gender	
Male	83
Female	31
T stage	
T1	50
T2	17
T3	41
T4	6
Grade	
G1	37
G2	69
G3	8
Histological type	
Clear cell	100
Non-clear cell	14

Results

Both RM1 and RM2 demonstrated a membranous, cytoplasmic staining pattern in all positive cells. In normal kidney, RM1 and RM2 stained distal tubules and the loop of Henle, but not the proximal tubules or glomeruli.

The expression of RM1 antigen (MSGb5) was detected in 59 out of 114 (51.8%) cases and that of RM2 antigen (GalNAcDSLc4) was detected in 15 (13.2%) cases (Figure 1). Nine out of 15 (60%) RM2 antigen-positive cases *vs.* 31 out of 99 (31.3%) negative cases showed metastasis at initial diagnosis ($p=0.0419$). *De novo* metastasis in patients without metastasis at initial diagnosis had a tendency to be more frequent in the RM2 antigen-positive group (2 out of 6, 33.3%) than in the RM2 antigen-negative group (10 out of 68, 14.7%), although the difference did not reach statistical significance, probably due to the small number of patients in the former group. The cumulative incidence of metastasis was 73.3% (11 out of 15) in the RM2 antigen-positive group *vs.* 41.1% (41 out of 99) in the antigen-negative group ($p=0.0266$, Table IIB). There was no association between the incidence of metastasis and RM1 antigen-positive cases or RM1 antigen and/or RM2 antigen-positive cases (Table IIA, C).

RM2 antigen-negative patients had a better prognosis ($p=0.0399$, Figure 2B). Neither RM1 antigen nor RM2 antigen staining was associated with the tumor stage, grade or histological type.

Discussion

A previous study using fresh-frozen sections of 55 primary renal tumors demonstrated that the frequent expression of gangliosides in RCC patients was correlated with metastasis, with a positive rate for RM1 and RM2 of 58% and 33%, respectively (16). Here, we examined a larger cohort of 114 RCC patients and showed that the positive rate for RM1 and RM2 was 51.8% and 13.2%, respectively. The positive rate

Table II. *Association of ganglioside expression with the incidence of metastasis.*

	Incidence of metastasis		Cumulative incidence of metastasis
	at diagnosis	during follow-up	
(A) RM1			
RM1-positive	23/59 (39.0%)	6/36 (16.7%)	29/59 (49.2%)
RM1-negative	17/55 (30.9%)	6/38 (15.8%)	23/55 (41.8%)
p-value	N.S.	N.S.	N.S.
(B) RM2			
RM2-positive	9/15 (60.0%)	2/6 (33.3%)	11/15 (73.3%)
RM2-negative	31/99 (31.3%)	10/68 (14.7%)	41/99 (41.4%)
p-value	0.0419	N.S.	0.0266
(C) RM1 and/or RM2			
RM1- and/or RM2-positive	27/64 (42.2%)	7/37 (18.9%)	34/64 (53.1%)
RM1- and RM2-negative	13/50 (26.0%)	5/37 (13.5%)	18/50 (36.0%)
p-value	N.S.	N.S.	N.S.

Table III. *Characteristics of RM2 antigen-positive cases without metastasis at initial diagnosis.*

Unique Pt. No.	Age	Gender	T	G	Histological type	RM1	RM2	Metastasis during follow-up	Follow-up (months)	Present status
127	62	F	1a	2	non clear cell	(+)	(+)	(–)	135	NED
195	74	M	2	1	clear cell	(+)	(+)	(+)	43	alive
272	57	M	3a	2	clear cell	(–)	(+)	(+)	78	dead
287	35	M	1a	2	clear cell	(+)	(+)	(–)	75	NED
308	65	M	1a	1	clear cell	(+)	(+)	(–)	1	NED
316	70	M	1a	1	clear cell	(+)	(+)	(–)	66	NED

NED: no evidence of disease.

for RM1 was comparable to that in the previous study; however, that for RM2 was considerably lower. We believe that this difference was due to the tissues' internal properties, because there were no technical differences, including in the procedure and antibodies, between these two studies. The previous study showed that the cumulative incidence of metastasis detected at initial diagnosis and during the follow-up period was significantly higher in RM1 antigen- and/or RM2 antigen-positive cases. However, we found that only RM2 antigen positivity was associated with a higher incidence of metastasis ($p=0.0266$), although the RM2 antigen-positive rate was lower than in the previous study (Table II). This shows that the expression of RM2 antigen strongly indicates the metastatic potential of human RCC.

The function of RM2 antigen as an adhesion molecule was previously studied *in vitro*. The binding of TOS-1 renal cancer cells to lung alveolar tissue was correlated with the expression of RM2 antigen, and binding was inhibited by RM2 but not by RM1 antibody (19). RM2 antigen showed extensive clustering at the cell surface and the fraction isolated from TOS-1 cells contained transducer molecules c-Src and Rho A, which were co-immunoprecipitated with RM2 antigen through RM2 (20). Furthermore, RM2 antigen showed strong binding to siglec-7, which promotes metastasis to the lung, whereas RM1 antigen did not show clear binding (21). Taking into consideration these functional studies and our results, we consider RM2 antigen to be a better marker of metastasis than RM1 antigen.

RM2 antigen-positive patients had a significantly poorer prognosis. This decreased survival can be explained by the presence of metastasis in 9 out of 15 (60%) RM2 antigen-positive cases at the time of initial diagnosis. On the other hand, the *de novo* appearance of metastasis was also higher in this group (33.3%) *vs.* the negative group (14.7%), although the difference was not statistically significant, probably to the limited number of patients. The cumulative incidence of metastasis was higher in RM2 antigen-positive

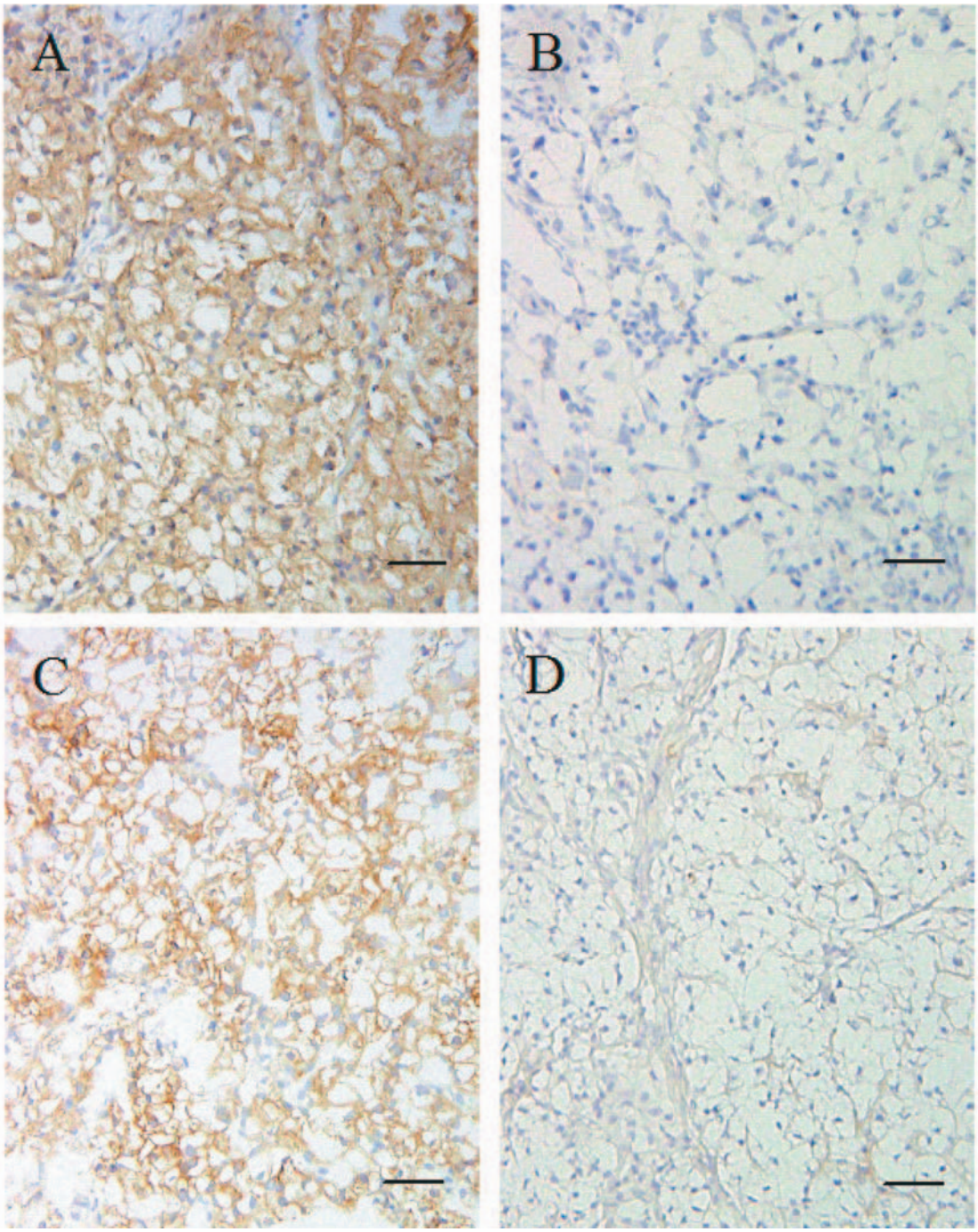

Figure 1. *Immunohistochemical staining using RM1 (MSGb5) A) positive (No. 149), B) negative (No. 60), RM2 (GalNAcDSLc4), C) positive (No.121), D) negative (No. 149). Scale bar is 50 μm long.*

cases (p=0.0266). Furthermore, there were 4 out of 6 RM2 antigen-positive tumors in stage T1 among patients without metastasis at initial diagnosis, and none of these 4 patients showed metastasis during the follow-up period. The remaining 2 patients had stage T2 and T3 tumors, and both developed metastasis during the follow-up period (Table III). In the RM2 antigen-negative cases, 10 out of 68 patients without metastasis at initial diagnosis showed metastasis during the follow-up period. These 10 cases were related to a high T stage and grade (p=0.0091, p=0.0004, respectively).

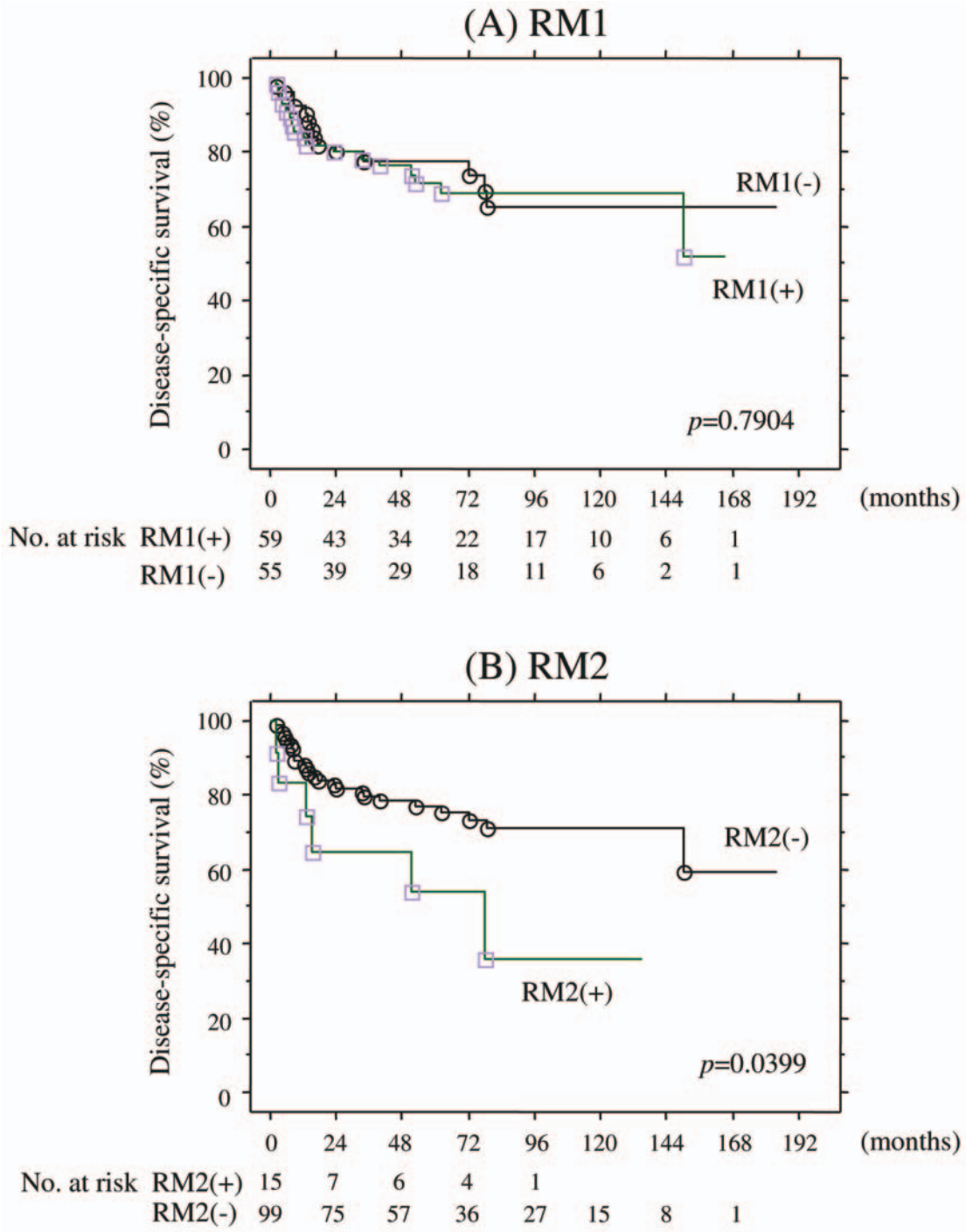

Figure 2. *Disease-specific survival of RCC patients according to A) RM1 (MSGb5) staining pattern, B) RM2 (GalNAcDSLc4) staining pattern; RM2 (GalNAcDSLc4) antigen-positive patients showed a significant decrease in survival.*

These results suggest that the RM2 antigen is not a better predictor than stage or grade of the *de novo* appearance of metastasis during follow-up in patients without metastasis at the initial diagnosis.

Recently, Aoki *et al.* tried to clarify the functional role of RM2 antigen and tried to knock down it by transfecting short interfering RNA (siRNA) targeting an open reading frame sequence of beta 1,4GalNAc transferase (beta

1,4GalNAc-T) (22). The RM2 antigen level remaied unchanged, even though it contains the beta 1,4GalNAc epitope at the terminus (22). Further extensive study using these new techniques will clarify the function of RM2 antigen as an adhesion molecule.

In conclusion, RCC patients bearing RM2 antigen (GalNAcDSLc4) on tumor cells should be carefully and thoroughly examined for metastasis at diagnosis and, if the disease is at an advanced stage, also during follow-up.

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