Clinical Significance of *CD151* Gene Expression in Non-Small Cell Lung Cancer[1]

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ABSTRACT

Transmembrane 4 superfamily (TM4SF) is a recently described gene family, and TM4SF members are known to play roles in the signal transduction pathways and to regulate cell activation, development, proliferation, and motility. MRP-1/CD9, KAI1/CD82, and ME491/CD63, members of the TM4SF, have been reported to suppress tumor progression or metastasis. Previously, we showed that MRP-1/CD9 suppressed cell motility and metastatic potential to lungs. Moreover, reduction of *MRP-1/CD9* and *KAI1/CD82* gene expression was found to be a factor in a poor prognosis for patients with non-small cell lung cancer. However, among TM4SF, *CD151* is identical to an existing gene, *PETA-3*, which may promote tumor metastasis of malignant cells, and its expression may be involved in the malignant progression of cancer. The function of *CD151* is opposite that of the metastasis suppressor genes, *MRP-1/CD9* and *KAI1/CD82*. On the basis of these results, we used reverse transcription-PCR and immunohistochemical techniques for a retrospective study of *CD151* gene expression in tumor tissues from 145 lung cancer patients; 72 tumors were stage I, 29 stage II, 27 stage IIIA, and 17 stage IIIB. Whereas 86 patients had tumors positive for the *CD151* gene, 59 had tumors that were negative for the *CD151* gene. The overall survival rate of patients with *CD151*-positive tumors was much lower than that of *CD151*-negative patients (51.9% *versus* 73.1%; $P = 0.013$). Our findings suggest that high *CD151* gene expression in lung cancer may be associated with a poor prognosis. Assessment of CD151 could be instrumental for improvements in lung cancer diagnosis and therapies.

INTRODUCTION

TM4SF[3] is a recently described gene family, and TM4SF members are known to play roles in signal transduction pathways and to regulate cell activation, development, proliferation, and motility (1). Among TM4SF, MRP-1/CD9 (2), KAI1/CD82 (3), and ME491/CD63 (4) have been reported to modulate tumor progression or metastasis. In our previous reports, we showed that tumor cells overexpressing MRP-1/CD9 had reduced cell motility and metastatic potential (2). In addition, reduced MRP-1/CD9 expression was associated with a poor prognosis in breast cancer (5), lung cancer (6), and pancreatic cancer (7). These data suggest that MRP-1/CD9 expression may be associated with metastatic ability and the degree of malignancy. KAI-1/CD82 is also a member of TM4SF, and KAI-1/CD82 expression suppressed experimental metastasis of rat prostate tumor cells and reduced their motility (8). *KAI1/CD82* is considered a metastasis-suppressor gene of prostate cancer, and low levels of KAI1/CD82 expression have been reported to be involved in the malignant progression of prostate cancer (3). We also showed that reduced *KAI1/CD82* gene expression was an indicator of a poor prognosis in lung cancer (9), breast cancer (10), and pancreatic cancer (7). These findings also indicate that *KAI1/CD82* is an important gene in cancer metastasis and progression.

On the other hand, CD151, a TM4SF protein also known as SFA-1 and PETA-3 (11, 12), was recently reported to be the first member of the TM4SF to be linked to a positive effecter of metastasis (13). This is contrary to the function of the metastasis suppressor genes, *MRP-1/CD9* and *KAI1/CD82*. In the present study, we investigated the clinical biological function of CD151 as a member of TM4SF, using RT-PCR and immunohistochemical techniques to evaluate the gene and protein levels of CD151 in lung cancer tissues as a prognostic factor in predicting the clinical behavior of lung cancer.

PATIENTS AND METHODS

Clinical Characteristics of Patients. Tumor specimens were obtained from 145 of the initial 166 patients with NSCLC who underwent surgery at the Department of Thoracic Surgery of the Kitano Hospital, Tazuke Kofukai Medical Research Institute (Osaka, Japan) between March 1991 and February 1996. Because of advanced stage lung cancer (stage IV), which involves several ill-defined factors and includes distant metasta-

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[3] The abbreviations used are: TM4SF, transmembrane 4 superfamily; CD, cluster of differentiation antigen; RT-PCR, reverse transcription-PCR; NSCLC, non-small cell lung cancer.

ses, eight patients were excluded from this study. Six patients with two or more different types of cancers and seven patients who died of causes other than lung cancer were also excluded from the study. As a result, we investigated 145 patients with lung cancer up to stage IIIB, and all cases were classified according to the Tumor-Node-Metastasis classification of malignant tumors: 72 tumors were stage I, 29 were stage II, 27 were stage IIIA, and 17 were stage IIIB (14). The clinical records and histopathological diagnoses of all of the patients were fully documented. The salient clinical characteristics of the patients are presented in Table 1. This report includes follow-up data up to July 31, 2000. The median follow-up for all patients was 48.0 months (range, 15.9–104.0 months).

Tumor Specimens. To ascertain the presence of cancer cells, half of the fresh tissue specimen from each tumor was immediately embedded in OCT compound (Miles, Kankakee, IL), and frozen sections were cut on a cryostat to a thickness of 6 μm and immediately stained with H&E. The other half of a given tumor specimen containing only cancer cells was used for RT-PCR.

RT-PCR Analysis. Total cellular RNA was purified from fresh or frozen tumor tissues by the acid guanidinium thiocyanate procedure (15). The RNA concentration was determined by spectrophotometry and adjusted to a concentration of 500 ng/μl. First strand cDNA synthesis was performed using a cDNA synthesis kit as indicated by the manufacturer (Pharmacia, Piscataway, NJ) from 5 μg of total RNA. For PCR amplification, a 1-μl aliquot of the reaction was used. To ensure reproducible quantitative performance of the RT-PCR assay, we titrated the amount of starting cDNA and the number of amplification cycles for *CD151*. Subsequent experiments were performed using the parameters that allowed amplification of both *CD151* and β-*actin* DNA (as the internal control) within a linear range. On the basis of the nucleotide sequence of *CD151*, 5′-ATGGGTGAGTTCAACGAGAAG-3′ was used as the sense primer and 5′-TCAGTAGTGCTCCAGCTTGAG-3′ was used as the antisense primer (12). This *CD151* primer pair amplifies a 761-bp fragment (nucleotides 85–846). The reaction mixture was subjected to 20 PCR amplification cycles of 40 s at 94°C, 40 s at 60°C, and 90 s at 72°C. β-*Actin* amplification was used as internal PCR control (16) with 5′-GATATCGCCGCGCTCGTCGTCGAC-3′ as the sense primer and 5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′ as the antisense primer. The same conditions were used to amplify β-*actin* DNA. Tubes containing all ingredients except templates were included in all runs as negative reaction controls. Preparations of the *CD151* positive human endothelial cell line, ECV304, were used as positive controls (17). Ethidium bromide staining of the 1% agarose gel after migration of the PCR products identified a band of the expected size for the pair of primers designed. To ensure reproducibility, all PCR amplifications were performed in duplicate. Densitometric analysis of the photographic negatives was used for band quantification.

Specimen Classification Based on RT-PCR Results. The value obtained for *CD151* by densitometry in a given sample was divided by that of β-*actin* and referred to as the *CD151* expression ratio. The expression ratio of the primary tumor was then divided by that of the human endothelial cell line, ECV304, which was used as a control to obtain the relative rate of *CD151* expression (17).

Table 1 Relationship between CD151 gene expression and various clinical and pathological variables

Variables	Total (*n*)	CD151 Positive (*n*)	CD151 Negative (*n*)	*P*
Gender				
F	35	21	14	NS[a]
M	110	65	45	
Age (yr)				
>60	101	60	41	NS
≤60	44	26	18	
Tumor status				
T_1	36	20	16	NS
T_2	73	42	31	
T_3	27	19	8	
T_4	9	5	4	
Nodal status				
N_0	91	51	40	NS
N_1	22	14	8	
N_2	23	15	8	
N_3	9	6	3	
Pathological status				
I	72	43	29	NS
II	29	15	14	
IIIA	27	19	8	
IIIB	17	9	8	
Histology				
AD	88	53	35	NS
SQ	49	28	21	
LA	8	5	3	
Differentiation				
Well	24	11	13	NS
Moderately	74	44	30	
Poorly	47	31	16	
Smoking				
Smoker	102	63	39	NS
Nonsmoker	43	23	20	
Total	145	86	59	

[a] NS, not significant; AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large cell carcinoma.

Immunohistochemical Assays. The assays were carried out as described previously (5). Endogenous peroxidases were blocked by incubation with 0.3% H_2O_2 in absolute methanol for 30 min. The sections were then incubated with 5% BSA for 2 h at room temperature, followed by incubation of replicate sections for 2 h with the anti-CD151 monoclonal antibody SFA1.2B4, three washings in PBS, and incubation for 1 h with biotinylated horse antimouse IgG (Vector Laboratories Inc., Burlingame, CA). Antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride, and the sections were lightly counterstained with Mayer's hematoxylin. As negative reaction controls, sections were incubated with mouse myeloma SP2 supernatant.

All immunostained sections were reviewed by two pathologists who had no knowledge of the patients' clinical status.

Statistical Analysis. The overall cancer-specific survival was defined as the period from the date of operation to the date of cancer death. The statistical significance of the difference between the incidence of *CD151*-positive expression and several clinical and pathological parameters was assessed with the

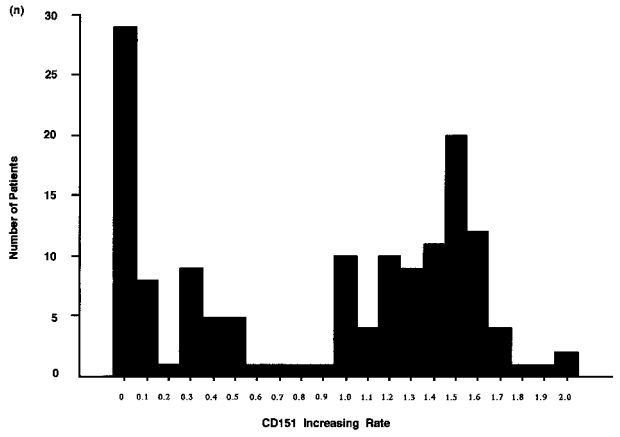

Fig. 1 Distribution of relative *CD151* expression (*increasing rate*). The values were obtained from the expression ratio of a given sample divided by that of a human endothelial cell line, ECV304, used as a positive control.

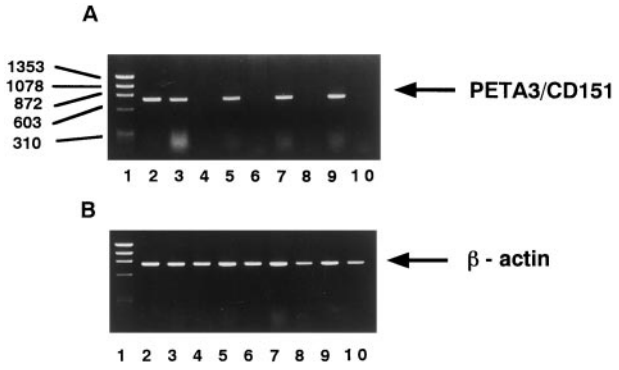

Fig. 2 *A*, agarose gel electrophoresis of RT-PCR-amplified 1031-bp *CD151* cDNA. *Lane 1*, size marker; *Lane 2*, human endothelial cell line ECV304 (positive control); *Lanes 3* and *5*, primary adenocarcinoma of lung with *CD151* gene expression evaluated as positive; *Lanes 4* and *6*, primary adenocarcinoma of lung with *CD151* gene expression evaluated as negative; *Lanes 7* and *9*, primary squamous cell carcinoma of lung evaluated as *CD151* gene positive; *Lanes 8* and *10*, primary squamous cell carcinoma of lung evaluated as *CD151* gene negative. *B*, agarose gel electrophoresis of amplified β-*actin* DNA (internal PCR control) of each of the specimens.

χ^2 test or the Mann-Whitney *U* test. The survival curves were estimated with the Kaplan-Meier method, and the resulting curves were compared using the log-rank test (18, 19). The joint effect of covariables was analyzed with the aid of the stepwise Cox proportional hazards regression model (SAS Institute, Cary, NC; Ref. 20), and two factors (nodal status and CD151 status of the tumor) were studied by assigning scores to each of the variables for the regression analysis. All *P*s were based on two-tailed statistical analysis and $P < 0.05$ was considered to indicate statistical significance.

Table 2 Relationship between RT-PCR results and immunohistochemical results of CD151

	RT-PCR		
Immunohistochemistry	*CD151* positive (*n*)	*CD151* negative (*n*)	Total (*n*)
CD151 positive	76	10	86
CD151 negative	3	56	59
Total	79	66	145

RESULTS

***CD151* Expression in Lung Tumor Tissues Analyzed by Quantitative RT-PCR.** The relative expression rates for *CD151* ranged from 0 to 2.0 with a distribution as shown in Fig. 1. The mean relative expression rate for *CD151* was 0.886, and the distribution of the relative expression rate was bimodal. Because most prognostic factors are usually considered as dichotomized, discontinuous variables, a cutoff point was selected to give the optimal separation between low and high risk for overall survival as described previously (21). Values >0.7 for the *CD151* relative expression rate in a given specimen were considered as indicating positive gene expression, values ≤0.7 were considered as denoting negative expression. Of the 145 primary lung cancers studied, a total of 86 carcinomas (59.3%) were evaluated as positive for *CD151* and 59 (40.7%) as negative for *CD151* (Fig. 2). Although 22 tumors showed only reduced *CD151* gene expression ($0.1 <$ *CD151* relative expression rate ≤ 0.7), the remaining 37 were quite negative for *CD151* (*CD151* relative expression rate ≤ 0.1).

CD151 Protein Expression Analyzed by Immunohistochemistry. When ≥50% of the tumor cells in a given specimen were positively stained, the sample was classified as positive, and when <50% of tumor cells were stained, it was classified as reduced. The RT-PCR cutoff point was almost consistent with the cutoff point (50%) for immunohistochemistry (Table 2). Of the 145 lung cancers studied with the immunohistochemical method, 79 (54.5%) were classified as CD151 positive (Fig. 3*A*). In these cases, CD151 immunostaining expression was intense and uniform on the surface of fixed, nonpermeabilized HEp-3 cells as seen in our study. Furthermore, with the aid of two-color confocal microscopy, we found similar localization of CD151 and integrin chains within cytoplasmic vesicles and endothelial cell junctions (13). Our study identified 66 cases (45.5%) with negative CD151 expression (Fig. 3*B*), and immunostaining of most of these tumors was heterogeneous. CD151 gene expression was readily evident in the primary tumors that were classified as positive in the immunohistochemical assays. In contrast, CD151 gene expression was weak or entirely absent in the lung cancers that immunohistochemically showed negative CD151. Overall, the immunohistochemical results showed good agreement (91.0%) with those of the RT-PCR assays (Table 2). In cases of discrepancy, the RT-PCR results were used for specimen classification.

Relationship between *CD151* Gene Expression and Clinical and Pathological Variables. We analyzed the relationships of *CD151* gene expression with several clinical and pathological variables. However, there were no statistically significant relationships by the χ^2 test and the Mann-Whitney *U* test between gene expression and the patients' gender, age, tumor size, lymph node status, histological subtypes, grade of differentiation, or smoking history (Table 1).

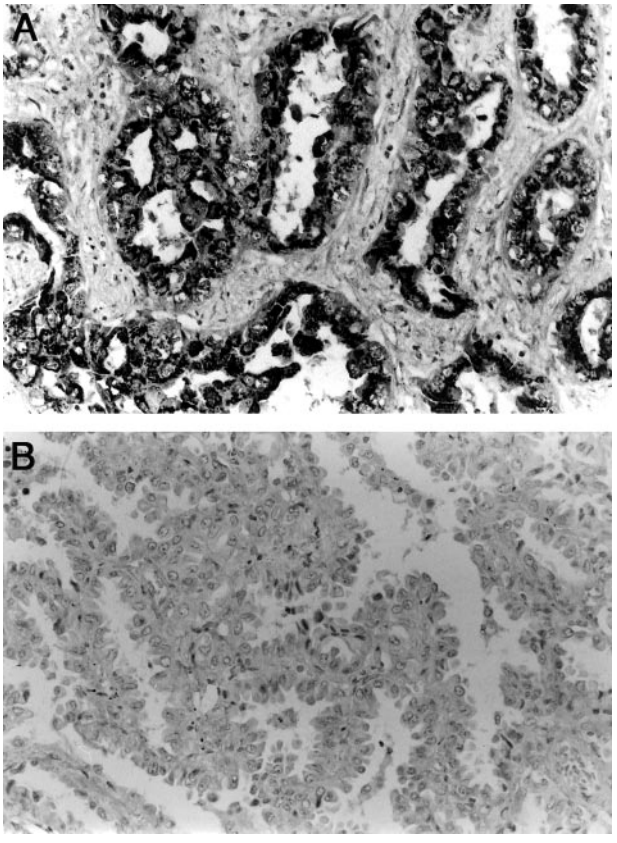

Fig. 3 *A*, CD151 expression in adenocarcinoma of the lung is intense and is seen uniformly at the cell surface and cytoplasm (positive staining). *B*, negative immunostaining of adenocarcinoma.

Table 3 Five-year survival rate of 145 patients with NSCLC in relation to clinical and pathological variables

Variables	5-year survival rate (%)	*P*
Gender		
F	65.1	0.227
M	58.7	
Age, years		
>60	63.2	0.309
≤60	53.3	
Tumor status		
T_1	69.8	0.071
T_2	61.2	
T_3	51.6	
T_4	37.5	
Nodal status		
N_0	70.3	0.007
N_1	53.1	
N_2	34.2	
N_3	41.7	
Pathological status		
I	75.4	<0.0001
II	57.2	
IIIA	36.6	
IIIB	35.2	
Histology		
AD[a]	56.4	0.433
SQ	62.5	
LA	66.9	
Differentiation		
Well	70.4	0.608
Moderately	58.3	
Poorly	57.7	
Smoking		
Smoker	60.6	0.575
Nonsmoker	60.1	
CD151		
Positive	51.9	0.013
Negative	73.1	

[a] AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large cell carcinoma.

Association of *CD151* Gene Expression with 5-Year Survival in Lung Cancer. When we compared the survival among all 145 patients according to *CD151* gene expression, the 5-year survival rate for patients with *CD151* gene-positive tumors was much lower than that of those with *CD151* gene-negative tumors (Table 3 and Fig. 4). The 5-year survival rate for the 86 patients with *CD151*-positive tumors was 51.9%, and that of the 59 patients with *CD151*-negative tumors was 73.1% ($P = 0.013$). Similarly, the patients with adenocarcinomas showed the same tendency for *CD151*-positive tumors to be associated with a progressive reduction in overall survival compared with those with negative tumors (47.4% *versus* 71.9%; $P = 0.047$). No such difference was found among the patients with squamous cell carcinomas.

Prognostic Value of *CD151* Status. The variables used for the Cox regression analysis are shown in Table 4. The estimated prognostic value of each of the variables in relation to the overall survival is expressed as *P*. Both variables (nodal status and *CD151* gene expression) were found to be significant factors for survival; *CD151* was significant for overall survival ($P = 0.0252$).

DISCUSSION

CD151 is a member of the TM4SF of cell-surface proteins and is identical to cDNA clones from the megakaryoblastic leukemia cell line designated 14A2.H1 (11), which was independently cloned from the adult T-cell leukemia line SF-HT and termed SFA-1 (SF-HT-activated gene 1; Ref. 12). The *CD151* cDNA clone shows an open reading frame that encodes a 253-amino acid protein with a molecular mass if 28 kDa (11). The human *CD151* gene is a single gene located on chromosome 11p15.5 (22) and has been reported to regulate platelet aggregation and mediator release (11, 23, 24). CD151 is expressed by a variety of cell types, including the basal cells of the epidermis; epithelial cells; skeletal, smooth, and cardiac muscle; Schwann cells; platelets; and endothelial cells (25), and has been shown to be associated with $\alpha 3\beta 1$ integrin in several cell types (26) as well as with the $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins (27, 28). Anti-CD151 monoclonal antibody inhibits endothelial cell migration and modulates *in vitro* angiogenesis (29). In addition, surprisingly, HeLa cells transfected with and overexpressing CD151 were found to be more migratory than control transfectants expressing little CD151 (13). CD151 belongs to a structurally distinct family of cell membrane glycoproteins, TM4SF, but it was reported to be identified to a positive effecter of metastasis for the human epidermoid carcinoma cell line HEp-3, the first member of TM4SF to be linked to the metastatic effecter (13).

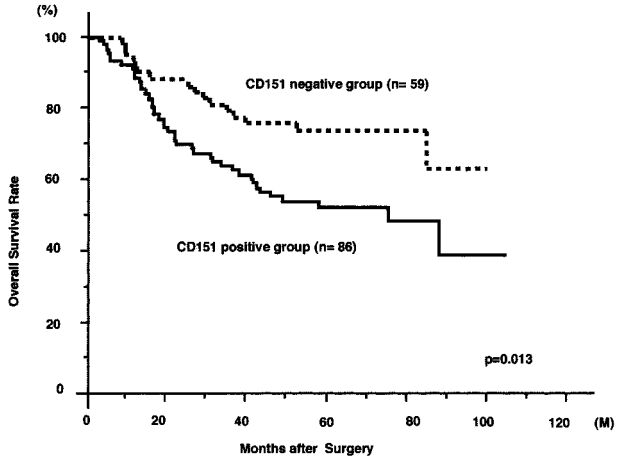

Fig. 4 Overall survival of 145 patients in lung cancer in relation to tumor *CD151* gene status. *P*s were determined with the log rank test.

Table 4 Multivariate regression analysis of variables for predicting overall survival of 145 patients with NSCLC

Variables	Assigned score	β	SE	Hazards ratio	95% CI[a]	*P*
CD151						
Negative	0	0.660	0.295	0.517	0.290–0.921	0.0252
Positive	1					
Nodal status						
N_1	1	0.401	0.125	1.493	1.1683–1.907	0.0014
N_2	2					
N_3	3					
N_4	4					

[a] CI, confidence interval.

As part of our evaluation of members of the TM4SF as possible prognostic predictors, we further extended our study to the expression of *CD151* and performed a retrospective study of the expression of the *CD151* gene by tumors of the lung. Subsequently, we demonstrated that *CD151* gene expression was associated with a poor prognosis for patients with NSCLC, especially in the case of adenocarcinoma. The patterns of recurrence indicate that distant metastases remain the primary cause of death in patients treated with surgery alone or surgery with chemotherapy and that these patterns are different for various histological types (30–32). In fact, distant metastases are by far the most common type of recurrence in patients treated for adenocarcinoma of the lung (33). In contrast, the incidence of local and distant recurrence was found to be equal in patients with squamous cell carcinoma, which in view of our earlier data is a very interesting phenomenon. Previously we were able to show that reductions in *MRP-1/CD9* and *KAI1/CD82* were likely to represent poor prognoses for patients with lung cancer and that the overall survival rate of *MRP-1/CD9*- and *KAI1/CD82*-positive patients with adenocarcinomas was much better than that for patients with reduced *MRP-1/CD9* or *KAI1/CD82* (34). These results represent a correlation that is the reverse of that established for *CD151*. Nevertheless, the gene status of the TM4SF members *CD151*, *MRP-1/CD9*, and *KAI1/CD82* appears to have prognostic relevance for lung cancer patients with adenocarcinoma.

Our data showed that CD151 was lost to the same degree at all four stages of lung cancer. Metastatic advances by tumor cells have been shown to depend on initial arrest in the secondary organ (35), as well as events that occur after extravasation, such as migration through the stroma to sites of preferred secondary tumor growth (36). Testa *et al.* (13) reported that CD151 is involved in an early step in the formation of metastatic foci, such as arrest, extravasation, and/or migration into the connective tissue stroma of the secondary organ. It is well known that an accumulation of genetic alterations causes the progression of tumors (37), but there have been very few reports on the relationship between the mechanism of metastasis and an accumulation of gene alterations in NSCLC. Many studies have reported that the complex composed of integrin α3, α5, α6, or β1 and TM4SF members might play an important role in cell attachment. There is the notion that the complex might have a significant role in maintaining the integrity of normal cells. Hence, it might be necessary to investigate such an association in normal tissues. Considering the progression of malignant tumors, aberrant complex composed of integrin α3, α5, α6, or β1 and CD151 might also occur even in the early stage of these tumors. On the other hand, as tumor malignancy proceeds, the association between integrins and KAI1/CD82 may diminish rapidly. During the last stage, the levels of MRP-1/CD9 might be reduced as a result of a aberrant methylation of the promoter, or aberrant glycosylation might occur in the first hydrophilic region, leading to a possible loss of the normal functions of MRP-1/CD9 (35). Thus, the malignant cells could eventually acquire metastatic potential. The decline in *MRP-1/CD9* expression might occur later than that of aberrant *CD151* gene expression. These and other questions about the functions of CD151, MRP-1/CD9, and KAI1/CD82 in various kinds of cancers may well be answered in the near future. Although the precise biological roles of CD151, MRP-1/CD9, and KAI1/CD82 have not been determined, our previous studies together with our present results suggest that aberrant expression of these genes appears to be associated with a poor prognosis in lung cancer and that these genes may therefore be potentially useful prognostic markers of metastasis. However, large-scale prospective and retrospective studies on various types of cancers are needed to establish whether CD151 is indeed of practical utility as a prognostic predictor. We need to establish the cell line and demonstrate the function of the TM4SF in the process of tumor metastasis. Thus, to reveal the process of metastasis related to the TM4SF, we are trying to clarify how the TM4SF affects cancer cell motility. We will report the result of this study in the near future.

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