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Fascin-1 overexpression and *miR-133b* downregulation in the progression of gastrointestinal stromal tumor

Hidetaka Yamamoto, Kenichi Kohashi, Aya Fujita and Yoshinao Oda

[1]*Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan*

MicroRNAs (miRNAs) are small, non-coding RNAs that are up- or downregulated in several types of cancer, and have an important role in the tumorigenesis and progression. To better understand the role of aberrantly expressed miRNAs and their target genes affecting the biology of gastrointestinal stromal tumor (GIST), we performed miRNA array in 19 cases of GIST, and found that several miRNAs, including *miR-133b*, were downregulated in high-grade GISTs. Subsequently, quantitative real-time reverse transcription-PCR revealed that *fascin-1* mRNA was upregulated in accordance with *miR-133b* downregulation in high-grade GIST; this result was consistent with a previous report showing that *fascin-1* might be a direct target of *miR-133b*. We then examined the fascin-1 protein expression by immunohistochemical staining in 147 cases of GIST, and found that fascin-1 overexpression was significantly correlated with shorter disease-free survival time and several aggressive pathological factors, including tumor size, mitotic counts, risk grade, blood vessel invasion and mucosal ulceration. Our results suggest that downregulation of *miR-133b* and overexpression of fascin-1 may have an important role in the progression of GIST, and that fascin-1 may be a useful biomarker to predict the aggressive behavior.
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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the alimentary tract.[1,2] The gain-of-function mutation of the *KIT* or *PDGFRA* gene is a key molecular event for development of GIST, and tyrosine kinase inhibitors targeting KIT and PDGFRA oncoproteins are in clinical use for advanced tumors.[3–6] GISTs exhibit a wide range of biological behaviors from benign to malignant, and the histological grade as defined by a combination of tumor size and mitotic counts is clinically useful to predict the patient prognosis.[1,2,7,8] In addition, the presence of blood vessel invasion[9] and mucosal ulceration[7] are also associated with the aggressiveness of GIST. However, the molecular mechanism for the progression of GIST has not been fully clarified.

Correspondence: Dr H Yamamoto, MD, PhD, Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan.
E-mail: hidetaka@surgpath.med.kyushu-u.ac.jp
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MicroRNA (miRNA) is a small (21–25 nucleotides) non-coding RNA that has important regulatory roles in cell proliferation, differentiation and death.[10,11] miRNAs bind through partial sequence homology to the 3′ untranslated region of target messenger RNAs (mRNAs) and inhibit the target genes by either blocking translation or promoting mRNA degradation. Aberrant expression of miRNAs has been reported in several types of human cancer; upregulated miRNAs in cancer may function as oncogenes by negatively regulating tumor-suppressor genes, whereas downregulation of some miRNAs in cancer may lead to upregulation of oncogenes.[12,13] There is growing evidence to suggest that unique miRNA expression profiles for each cancer type would be a useful biomarker for cancer diagnosis and prognosis.[12,14] As for GISTs, miRNA expression studies have revealed that aberrant expression of miRNAs is related with 14q deletion, a common chromosomal abnormality in GIST.[15,16] A very recent study reported that upregulation of miR-199a was correlated with high-risk-grade and worse prognosis in patients with GISTs.[17] However, further analysis is needed to clarify the role of miRNAs in the development and progression of GIST.

In this study, we identified several miRNAs that were significantly downregulated in high-grade GISTs. Among such miRNAs, *miR-133b* has been reported to be downregulated in some kinds of cancer, including esophageal squamous cell carcinoma, and *miR-133b* can directly regulate the expression of *fascin-1* (*fascin homolog-1, FSCN1*, also called *fascin*), an oncogenic actin-binding protein in esophageal squamous cell carcinoma.[18] These finding prompted us to elucidate the potential clinicopathological significance of fascin-1 expression in GIST.

Materials and methods

Case Materials

A total 147 cases of primary GIST were obtained from the files of the Department of Anatomic Pathology, Kyushu University, Fukuoka, Japan. None of the cases were treated with imatinib before the initial surgical operation. Each GIST was evaluated for clinicopathologic and histologic features, including tumor size, mitotic counts, blood vessel invasion and mucosal ulceration. Mitoses were counted and summed from 50 high-power fields (HPFs). Blood vessel invasion was evaluated according to our previous study.[9] The grade of each tumor was determined by a combination of tumor size and mitotic counts based on two classification systems: the system proposed by Fletcher *et al*[1] at a National Institutes of Health (NIH) consensus meeting and that proposed by Miettinen *et al*[2] at the Armed Force Institute of Pathology (AFIP). Immunohistochemical staining was performed in 147 cases, and prognostic data with long follow-up time were available in 105 cases.

Among the 147 cases, identical snap-frozen samples maintaining a sufficient quality of RNA for microarray study were also available in 19 cases.

This study was approved by the Surveillance Committee of Kyushu University (no.24–24).

KIT and *PDGFRA* Gene Mutation Analysis

Genomic DNA was extracted from snap-frozen samples by using standard proteinase K digestion and phenol/chloroform extraction. Mutations in exons 9, 11, 13 and 17 of the *KIT* gene and those in exons 12 and 18 of the *PDGFRA* gene were examined in 19 cases of GIST, as previously reported.[6] We further classified KIT genotype into aggressive genotype (KIT exon 11 deletion or KIT exon 9 duplication) and indolent genotype (KIT exon 11 missense mutation, KIT exon 11 internal tandem duplication or KIT wild), because GISTs with KIT exon 11 deletion or KIT exon 9 duplication have more aggressive biological behavior than those with other genotypes (reviewed in ref. Lasota and Miettinen[3]).

RNA Extraction and miRNA Expression Profiling

Total RNA, including small RNAs, was extracted from the snap-frozen tissues by using miRNeasy mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Before microarray analysis, electrophoresis of RNA was performed to check the quality of RNA. Nineteen samples of GISTs that clearly showed peaks for 18srRNA and 28srRNA were considered to have RNA of sufficient quality for microarray study. Extracted total RNA was labeled with Hy5 using the miRCURY LNA Array miR labeling kit (Exiqon, Vedbaek, Denmark). Labeled RNAs were hybridized onto 3D-Gene Human miRNA Oligo chips containing duplicate spots of anti-sense probes for 904 human miRNAs and 107 viral miRNAs (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes were confirmed by using the miRBase miRNA database (http://microrna.sanger.ac.uk/sequences/). After stringent washes, fluorescent signals were scanned with the ScanArray Express Scanner (PerkinElmer, Waltham, MA, USA) and analyzed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). The raw data of each spot were normalized by substitution with a mean intensity of the background signal determined by all blank spots' signal intensities of 95% confidence intervals. Measurements of both duplicate spots with the signal intensities >2 s.d. of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalization by their median values adjusted equivalently. miRNAs differentially expressed depending on the pathological parameters were statistically identified using *t*-test. Raw data from the microarray analysis are available on the website of the Gene Expression Omnibus (accession no. GSE36087, http://www.ncbi.nlm.nih.gov/geo/).

Quantitative Real-Time RT-PCR for *miR-133b*

We examined the *miR-133b* expression level by quantitative real-time reverse transcription-PCR (RT-PCR) to validate the microarray results. In brief, total RNA was extracted as mentioned above, and cDNA was produced by reverse transcription with miScript Reverse Transcription kit (QIAGEN) according to the manufacturer's instructions. By using ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), we performed quantitative PCR for *miR-133b* and *SCARNA17*; the latter was an endogenous control. The PCR reagent for each sample contained 50 ng of cDNA, 25 μl of QuantiTect SYBR Green PCR Master Mix, 5 μl of miScript Universal Primer as a reverse primer and 5 μl of miScript Primer Assay for either *miR-133b* (MS00031430) or *SCARNA17* (MS00014014) as a

Table 1 Clinicopathological and molecular findings of 19 cases of GIST available for miRNA analysis

No.	*Age*	*Sex*	*Site*	*Size (cm)*	*Mitosis (per 50 HPF)*	*NIH grade*	*AFIP grade*	*KIT mutation*
1	71	M	Small intestine	8.5	6	High	High	Wild
2	79	F	Stomach	5.3	1	Intermediate	Low	KIT exon 11 missense
3	66	M	Small intestine	5.5	1	Intermediate	Moderate	Wild
4	88	M	Large intestine	18	25	High	High	Wild
5	74	F	Stomach	17	28	High	High	KIT exon 11 deletion
6	53	F	Stomach	4	2	Low	Low	Wild
7	60	M	Small intestine	27	6	High	High	KIT exon 11 deletion
8	58	M	Stomach	5	112	High	Moderate	KIT exon 11 deletion
9	38	M	Small intestine	13	2	High	High	KIT exon 11 deletion
10	49	M	Stomach	6.5	0	Intermediate	Low	Wild
11	40	M	Stomach	6.5	121	High	High	KIT exon 11 deletion
12	73	F	Stomach	3.8	2	Low	Low	KIT exon 11 deletion
13	76	M	Small intestine	7.2	25	High	High	KIT exon 11 deletion
14	68	M	Stomach	6.8	2	Intermediate	Low	Wild
15	70	F	Stomach	3.5	0	Low	Low	KIT exon 11 duplication
16	47	M	Small intestine	5.5	18	High	High	Wild
17	46	F	Small intestine	6	10	High	High	KIT exon 9
18	60	M	Stomach	4	4	Low	Low	Wild
19	69	F	Large intestine	5	13	High	High	KIT exon 11 deletion

forward primer. The cycle conditions can be seen in the manufacturer's instructions (http://www.qiagen.com/). Standard curves were generated using serial dilutions of the cDNA samples of the non-tumor tissue of the gastrointestinal wall. The quantity of miRNAs in each GIST sample was estimated by comparison with standard curves, and the ratio of the quantity of *miR-133b* to that of *SCARNA17* was used as a relative measure of the *miR-133b* expression level in each GIST specimen.

Quantitative Real-Time RT-PCR for *Fascin-1* mRNA

As we hypothesized that *fascin-1* might be a candidate target of *miR-133b* in GIST just as in esophageal squamous cell carcinoma (see Result section), we examined the *fascin-1* mRNA level by quantitative real-time RT-PCR and compared it with the *miR-133b* expression level in 19 cases of GIST. In brief, the PCR reagent for each sample contained 50 ng of cDNA created as described above, 25 μl of QuantiTect SYBR Green PCR Master Mix and 5 μl of QuantiTect Primer Assay containing both forward and reverse primers for either *fascin-1* (QT00019747; QIAGEN) or *β-actin* (QT01680476; QIAGEN); the latter was used as an endogenous control. The relative mRNA expression level of *fascin-1* in each GIST specimen was calculated by the ratio of *fascin-1* quantity to *β-actin* quantity.

Immunohistochemistry for Fascin-1

Immunohistochemical staining was performed on the formalin-fixed and paraffin-embedded specimens with the primary antibodies against fascin-1 (mouse monoclonal, clone: 55K-2; dilution: 1/50; Dako Cytomation, Carpinteria, CA, USA). The proportion and intensity of cytoplasmic immunoreactivity in tumor cells were evaluated. The proportion was scored according to the percentage of fascin-immunoreactive tumor cells as follows: negative (0%), 0; focal (1–50%), 1; and diffuse (>50%), 2. The intensity was scored as negative, 0; weak, 1; and strong, 2. In each slide, endothelial cells showed cytoplasmic expression of fascin-1, and immunoreactivity equal to or stronger than that in endothelial cells was considered as strong positivity. A total score of 0 (negative expression) or 2 (focal and weak expression) was designated as 'low expression,' and a score of 3 (focal and strong expression or diffuse and weak expression) or 4 (diffuse and strong expression) was designated as 'high expression'.

Statistical Analysis

The correlation between the expression level of *miR-133b* or *fascin-1* mRNA and clinicopathological parameters was analyzed by Pearson's correlation test or Mann–Whitney *U*-test. The correlation between the fascin-1 protein level and clinicopathological factors was analyzed by the χ^2 test. For univariate and multivariate analyses of disease-free survival, we used the Kaplan–Meier method with the log-rank test and the Cox proportional hazard model, respectively. A *P*-value of <0.05 was considered statistically significant.

Results

Clinicopathological Findings and Mutations in *KIT* and *PDGFRA* Genes

The clinicopathological findings and results of mutation analysis in 19 cases are summarized in Table 1. According to the NIH risk grade, 4 cases were classified as low grade, 4 as intermediate grade and 11 as high grade. The gene mutations were found in

Table 2 miRNAs downregulated in high-grade GIST

miRNA	*Ratio*[a]	*P-value*
hsa-miR-483-5p	0.49	0.0289
hsa-miR-1268	0.53	0.0001
hsa-miR-508-5p	0.56	0.0391
hsa-miR-1915	0.59	0.0001
hsa-miR-762	0.59	0.0008
hsa-miR-452	0.60	0.0447
hsa-miR-371-5p	0.61	0.0278
hsa-miR-638	0.62	0.0019
hsa-miR-744	0.65	0.0092
hsa-miR-1225-5p	0.66	0.0099
hsa-miR-1272	0.67	0.0013
hsa-miR-885-3p	0.68	0.0378
hsa-miR-137	0.71	0.0163
hsa-miR-133b	0.72	0.0486
hsa-miR-206	0.73	0.0026
hsa-miR-1261	0.73	0.0201
hsa-miR-939	0.74	0.0122
hsa-miR-572	0.75	0.0124
hsa-miR-767-3p	0.77	0.0142
hsa-miR-1228*	0.77	0.0008
hsa-miR-892b	0.78	0.0309
hsa-miR-589	0.78	0.0408
hsa-miR-149*	0.79	0.0180
hsa-miR-526b	0.79	0.0302

[a]Median of high-grade GIST/median of low-to-intermediate-grade GIST <0.8.
hsa-miR-133b is bolded, because this miRNA is examined in the current study.

KIT exon 9 in 1 case and *KIT* exon 11 in 10 cases (deletion, 8 cases; missense mutation, 1 case; internal tandem duplication, 1 case), whereas there were no mutations in either *KIT* or *PDGFRA* in 8 cases. The primary sites were stomach, small intestine and large intestine in 10, 7 and 2 cases, respectively.

Differently Expressed miRNAs Depending on Clinicopathological Factors: Supervised Analysis

By supervised analysis, we found that about 10–70 miRNAs were differently expressed depending on individual clinicopathological parameters, such as primary site, tumor size, mitotic counts, tumor grade and KIT genotype (data not shown). Table 2 shows 24 miRNAs that were downregulated in NIH high-grade GIST as compared with low-to-intermediate-grade GIST ($P<0.05$, median of the former/median of the latter <0.8). In order to narrow the candidate miRNAs related with tumor progression, we compared our data with that of a previous study by Choi *et al*.[15] We found that *miR-133b* was the only miRNA that was significantly downregulated in NIH high-grade GIST in both cohorts (Supplemental Table 4 of Choi *et al*[15]).

Quantitative Real-Time RT-PCR for *miR-133b* and *Fascin-1*

We examined the *miR-133b* expression level by quantitative real-time RT-PCR to validate the microarray result in 19 cases of GIST, and confirmed that *miR-133b* expression tended to be lower in NIH high-grade GISTs than in intermediate-to-low-grade GISTs ($P=0.0575$, Figure 1a). In addition, the *miR-133b* expression level was inversely correlated with mitotic counts ($P=0.0275$). The primary site, tumor size, AFIP grade and *KIT* genotype were not correlated with the *miR-133b* level ($P=0.5676$, $P=0.4829$, $P=0.1416$ and $P=0.3218$, respectively).

We then checked the predicted mRNA target of *miR-133b* by using TargetScan (http://www.targetscan.org/), and found 648 predicted targets, including *FSCN1* (*fascin-1*), *MCL1* (*myeloid cell leukemia sequence 1*) and *FLT1* (*vascular endothelial growth factor receptor*). As a previous study on esophageal squamous cell carcinoma revealed that *fascin-1* expression was actually regulated by *miR-133b*,[18] we decided to examine the correlation between *miR-133b* and *fascin-1* expression levels, and the clinicopathological significance of fascin-1 in GIST. By using quantitative real-time RT-PCR, the expression level of *miR-133b* was inversely correlated with that of *fascin-1* mRNA ($r=-0.47$, $P=0.0453$) in 19 cases of GIST (Figure 1b). The expression level of *fascin-1* mRNA was significantly correlated with the fascin-1 protein level as determined by immunohistochemical staining ($P=0.0003$; Figure 1c). Furthermore, higher expression of *fascin-1* mRNA was also significantly correlated with the NIH high-risk grade ($P=0.0064$; Figure 1d), AFIP high-risk grade ($P=0.0275$) and higher mitotic counts (>5/50 HPFs, $P=0.0114$), but not with the primary site (stomach *vs* intestine, $P=0.3272$) or tumor size (>5 *vs* ≤ 5 cm, $P=0.1144$). The *fascin-1* mRNA expression level was significantly higher in GISTs with aggressive KIT genotype (KIT exon 11 deletion or KIT exon 9 duplication) than those with indolent KIT genotype (KIT exon 11 missense mutation, KIT exon 11 internal tandem duplication or KIT wild; $P=0.0455$). However, it was not correlated with presence or absence of *KIT* mutation (any type of mutation *vs* wild, $P=0.1167$).

Fascin-1 Protein Expression and its Clinicopathological Significance

We extended the immunohistochemical and clinicopathological study to a total 147 cases of surgically resected GIST. The 147 patients comprised 74 men and 73 women, ranging in age from 20 to 93 years (median, 64 years). The tumor ranged from 1.2 to 27 cm in size (median, 5.8 cm). Mitotic counts varied from 0 to 121 per 50 HPFs (median, 3/50 HPFs). According to the NIH risk grade, 4 cases were classified as very low grade, 36 as low grade, 55 as intermediate grade and 52 as high grade. According to the AFIP risk grade, 35 cases were classified as very low grade, 35 as low grade, 34 as moderate grade and 43 as high grade. Primary sites

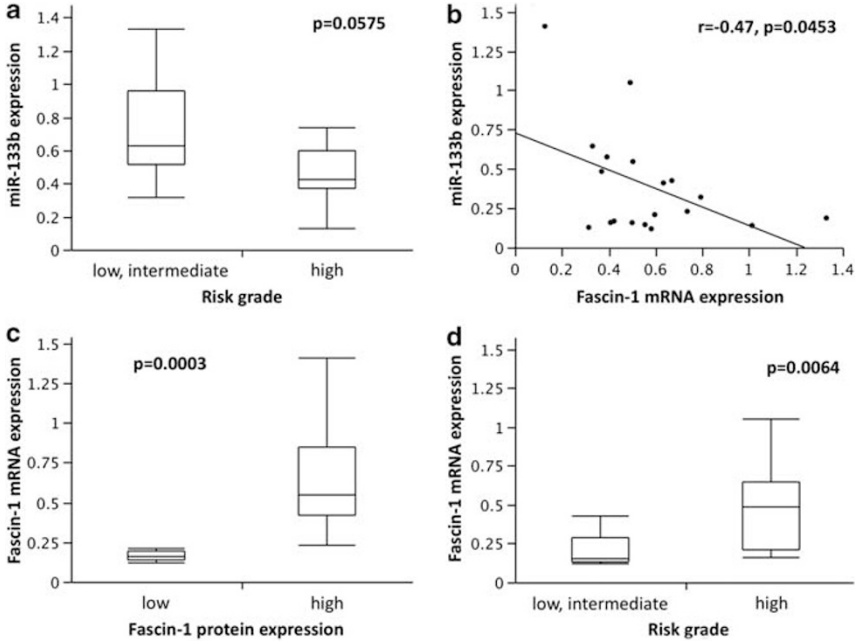

Figure 1 The expression levels of *miR-133b* and *fascin-1* mRNA determined by real-time RT-PCR. (**a**) *miR-133b* expression tended to be lower in high-grade GISTs than in intermediate-to-low-grade GISTs ($P=0.0575$). (**b**) The *miR-133b* expression level was inversely correlated with that of *fascin-1* mRNA ($r=-0.47$, $P=0.0453$). (**c**) The *fascin-1* mRNA level was significantly correlated with the fascin-1 protein level as determined by immunohistochemical staining ($P=0.0003$). (**d**) *Fascin-1* mRNA was significantly upregulated in NIH high-risk-grade GIST compared with the level in intermediate-to-low-grade GISTs ($P=0.0064$).

were stomach and intestine in 106 and 41 cases, respectively. Blood vessel invasion and mucosal ulceration were present in 29 and 50 cases, respectively (Figures 2a and b). Fascin-1 protein expression was classified as negative (total score 0; $n=77$), focal weak (total score 2; $n=30$), focal strong (total score 3; $n=2$), diffuse weak (total score 3; $n=14$) and diffuse strong (total score 4; $n=24$; Figures 2c and d). Therefore, 77, 30, 16 and 24 cases were scored as 0, 2, 3 and 4, respectively, which corresponded to low (total scores 0, 2) and high (total scores 3, 4) expressions in 107 (73%) and 40 (27%) cases, respectively. Table 3 summarizes the correlation between the fascin-1 protein expression level (low *vs* high) and clinicopathological parameters. High expression of fascin-1 was significantly correlated with larger tumor size ($P=0.0243$), higher mitotic counts ($P<0.0001$), NIH high grade ($P<0.0001$), AFIP high grade ($P<0.0001$) and presence of blood vessel invasion ($P<0.0001$) and mucosal ulceration ($P<0.0001$), but not with primary tumor site ($P=0.4156$).

Follow-up information was available in 105 cases. In all 105 cases, imatinib treatment was not performed before or after the initial surgery until recurrence and/or metastasis occurred. The cases with liver and/or peritoneal metastasis at the initial surgery were excluded from the prognostic analysis. Local recurrence or peritoneal metastasis occurred in 7 cases, and distant metastasis, which was liver metastasis in the vast majority of cases, was present in 15 cases after the initial surgery. A total of 17 cases (16%) of the primary localized GIST showed recurrence and/or metastasis after surgery. The prognostic values of the fascin-1 expression level and other clinicopathological parameters for disease-free survival were analyzed. High expression of fascin-1 (scores 3 and 4) was significantly correlated with shorter disease-free survival time as compared with low expression (scores 0 and 2, $P<0.0001$; Table 4, Figure 3a). In addition, larger tumor size (>5 cm, $P=0.023$), higher mitotic counts ($>5/50$ HPF, $P<0.0001$), NIH high grade ($P<0.0001$), AFIP high grade ($P<0.0001$) and presence of blood vessel invasion ($P<0.0001$) and mucosal ulceration ($P<0.0001$) were each correlated with worse prognosis in univariate analysis (Table 4, Figures 3b–d). The primary site (stomach *vs* intestine) did not influence the difference in the disease-free survival time. In multivariate analysis, tumor size, mitotic counts and blood vessel invasion were identified as independent worse prognostic factors for disease-free survival ($P=0.0455$, $P=0.0055$ and $P=0.0077$, respectively; Table 4).

Discussion

In this study, we examined the miRNA expression profile in GISTs and found that *miR-133b* was downregulated in high-grade tumors as compared with lower-grade ones, which was consistent with a previous report.[15] Recent studies have reported that *miR-133b*, mapped on chromosome 6p12.2, was downregulated in carcinomas in the

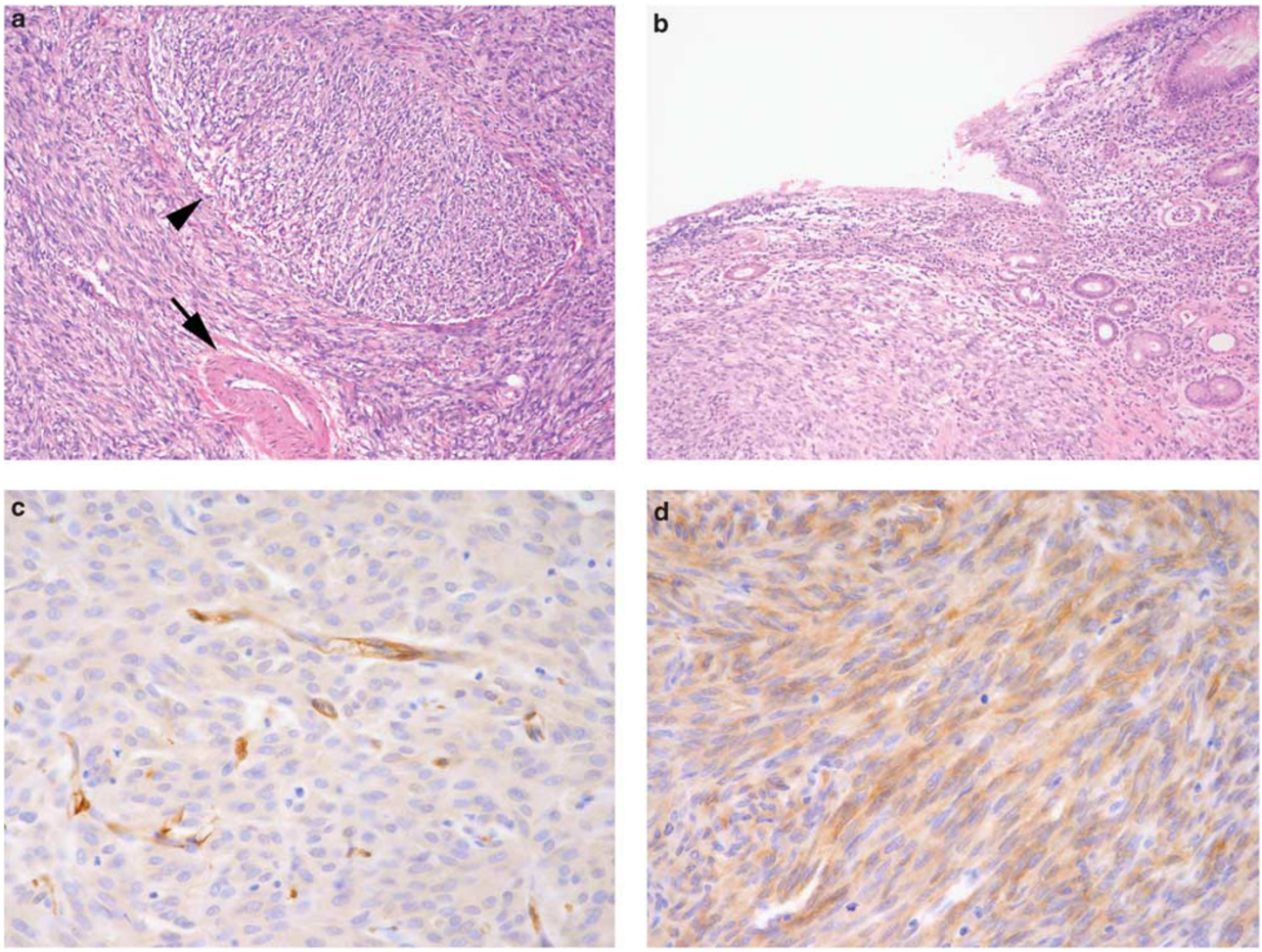

Figure 2 Histological findings (**a**, **b**) and immunohistochemical staining for fascin-1 (**c**, **d**) in GIST. (**a**) Blood vessel invasion. Tumor thrombus in the vein is indicated by an arrowhead, and the artery is indicated by an arrow. (**b**) Mucosal ulceration. Tumor cells invade the mucosa, and the mucosal epithelium disappears at the ulcer. (**c**) Weak expression of fascin-1. The immunoreactivity for fascin-1 in tumor cells is weaker than that in endothelial cells. (**d**) Strong expression of fascin-1. Tumor cells show consistent immunoreactivity for fascin-1.

urinary bladder, colon, lung and esophagus.[18–21] Downregulation of *miR-133b* in cancer cells seems to result in upregulation of target mRNA and thus, overexpression or activation of oncoprotein. In lung cancer, downregulation of *miR-133b* is correlated with upregulation of *MCL-1* and *BCL2L2*, suggesting an anti-apoptotic role.[21] Kano *et al*[18] reported that *miR-133b* was downregulated in esophageal squamous cell carcinoma clinical specimens as compared with the normal epithelium. Furthermore, they found that induction of *miR-133b* inhibited *fascin-1* expression in squamous cell carcinoma cell lines, and silencing of *fascin-1* function by siRNA inhibited both proliferation and invasion of squamous cell carcinoma cells, suggesting that *miR-133b* might be a tumor-suppressive miRNA, and directly control the oncogenic *fascin-1* gene.[18] Therefore, we hypothesized that downregulation of *miR-133b* might be associated with upregulation of *fascin-1* during the progression also in GIST.

Fascins are actin-binding proteins and are important for the maintenance and stability of parallel bundles of filamentous actin in a variety of cells.[22,23] The ability of fascin to bind and bundle actin has a central role in the regulation of cell adhesion and migration. Three subtypes of fascins are present in human tissue: fascin-1, fascin-2 and fascin-3. Fascin-1 is widely expressed in mesenchymal tissue and the nervous system, whereas fascin-2 and fascin-3 are specific to the retinal photoreceptor cells and testis, respectively.[23] Although fascin-1 expression is absent or very low in the normal epithelium, it is upregulated in several types of carcinomas, including those of the lung, esophagus, stomach, colon, pancreas and urinary bladder.[24–29] Furthermore, overexpression of fascin-1 is correlated with worse prognosis and metastasis in several types of carcinomas.[25–27] The role of fascin-1 in carcinomas also has been probed experimentally in cell lines and mouse models.[28,30–33] Collectively, these findings indicate that fascin-1 functionally

Table 3 The correlation between fascin-1 expression and clinicopathological parameters in GIST ($n=147$)

	Fascin-1 expression		P-*value*
	High (n = *40*)	*Low* (n = *107*)	
Site			
Stomach ($n=106$)	31	75	0.4156
Intestine ($n=41$)	9	32	
Size (cm)			
≤ 5 ($n=59$)	10	49	0.0243
>5 ($n=88$)	30	58	
Mitosis (per 50 HPF)			
≤ 5 ($n=92$)	11	81	<0.0001
>5 ($n=55$)	29	26	
NIH grade[a]			
Very low ($n=4$)	1	3	<0.0001
Low ($n=36$)	3	33	
Intermediate ($n=55$)	6	49	
High ($n=52$)	30	22	
AFIP grade[a]			
Very low ($n=35$)	4	31	<0.0001
Low ($n=35$)	2	33	
Moderate ($n=34$)	9	25	
High ($n=43$)	25	18	
Blood vessel invasion[b]			
Negative ($n=115$)	21	94	<0.0001
Positive ($n=29$)	18	11	
Mucosal ulceration			
Negative ($n=97$)	13	84	<0.0001
Positive ($n=50$)	27	23	

[a]Very low, low and intermediate/moderate grade *vs* high grade.
[b]Data for three cases are unavailable.

contributes to tumor progression, particularly in terms of invasion and metastasis.

The molecular mechanisms of transcriptional regulation of *fascin-1* have not been fully elucidated; however, the cAMP response element-binding protein and aryl hydrocarbon receptor are candidate-positive regulators that enhance the promoter activity of *fascin-1*.[23,34] In contrast, several miRNAs, such as *miR-133a, 133b, 143* and *145*, are thought to negatively regulate the *fascin-1* transcript in vascular smooth muscle cells[35] and carcinoma cells including urothelial carcinoma cells and squamous cell carcinoma cells.[18,36] These miRNAs have conserved sequences in the 3′ untranslated region of *fascin-1*. In this study, the expression level of *miR-133b* was inversely correlated with that of *fascin-1* mRNA in our series of clinical samples of GIST. However, we cannot jump to the conclusion that *miR-133b* is a major regulator of the *fascin-1*

Table 4 The univariate and multivariate analysis of prognostic factors in GIST ($n=105$)

	Disease-free survival	
	Univariate (P =)	*Multivariate* (P =)
Fascin-1 (high)	<0.0001	0.3911
Tumor size (>5cm)	0.023	0.0455
Mitosis (>5/50 HPF)	<0.0001	0.0055
NIH grade (high)	<0.0001	Not included
AFIP grade (high)	<0.0001	Not included
Blood vessel invasion (positive)	<0.0001	0.0077
Mucosal ulceration (positive)	<0.0001	0.4664

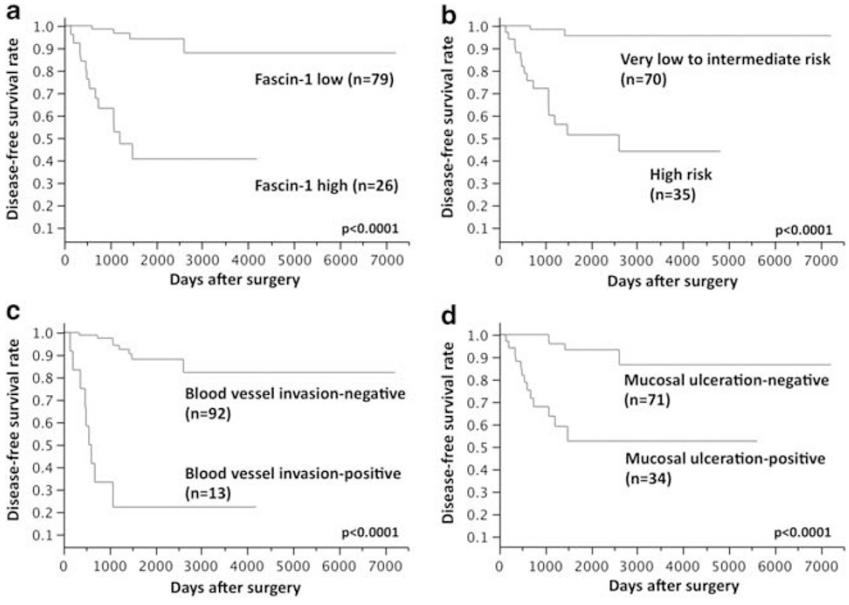

Figure 3 Kaplan–Meier analyses for 105 patients with primary localized GISTs. High expression of fascin-1 protein (**a**), NIH high grade (**b**), blood vessel invasion (**c**) and mucosal ulceration (**d**) are each significantly correlated with shorter disease-free survival time (each $P<0.0001$).

gene in GIST cells, because we have not performed a functional study using GIST cell lines. In addition, fascin-1 expression might also be regulated by the above-mentioned molecular mechanism, including miRNAs other than *miR-133b*.

Nevertheless, we found that the increased expression of fascin-1 protein was present in approximately one-quarter of GISTs, and it was significantly correlated with shorter disease-free survival time, higher rate of liver metastasis and several aggressive pathological factors, including tumor size, mitotic counts, risk grade, blood vessel invasion and mucosal ulceration. Our result is consistent with the function of fascin-1 as an oncoprotein, and suggests that fascin-1 may have an important role in the progression of GIST. This is the first report to show the prevalence and clinicopathological significance of fascin-1 expression in a large series of GIST. In a previous study examining several types of sarcomas, fascin-1 overexpression was frequently observed in follicular dendritic cell tumor, whereas it was present in only 1 of 13 cases (8%) of GIST.[37]

In our previous study, blood vessel invasion was significantly correlated with the high incidence of liver metastasis in GIST.[9] Overexpression of fascin-1 in GIST cells might increase the invasiveness of tumor cells and help them to infiltrate into blood vessels. In a series of patients with hepatocellular carcinoma, portal vein invasion and intrahepatic metastasis were found to be more prevalent in fascin-1-positive cases.[38]

Similarly, the presence of mucosal ulceration was correlated with both worse prognosis and fascin-1 overexpression in the current series of GIST. A previous study by Miettinen *et al*[7] also reported that ulceration represented the aggressive behavior of gastric GIST. These findings suggest that fascin-1 overexpression may contribute to invasion of GIST cells into the adjacent tissue and result in ulcer formation at the mucosa.

Although fascin-1 overexpression was correlated with worse prognosis in univariate analysis, it was not an independent prognostic factor in multivariate analysis (Table 4). One possible explanation for this discrepancy is that other prognostic factors may be confounding variables in multivariate analysis, because fascin-1 expression was strongly correlated with other prognostic factors such as tumor size, mitosis, risk grade, blood vessel invasion and mucosal ulceration (Table 3).

It is well known that GISTs with KIT exon 11 deletion or KIT exon 9 duplication have more aggressive biological behavior than those with other genotypes.[3,39] In this study, we classified KIT genotype into aggressive genotype (KIT exon 11 deletion or KIT exon 9 duplication) and indolent genotype (KIT exon 11 missense mutation, KIT exon 11 internal tandem duplication or KIT wild). Interestingly, the *fascin-1* mRNA level was significantly higher in GISTs with aggressive KIT genotype than in those with indolent KIT genotype ($P = 0.0455$), although limited number of cases were examined. The result is consistent with the fact that fascin-1 overexpression is correlated with aggressive behavior of GIST. Further study is needed to elucidate the possible correlation between KIT genotype, fascin-1 overexpression and biological behavior in larger series of GIST.

Recently, migrastatin was shown to block tumor metastasis by binding with fascin-1 and inhibiting fascin/actin bundling in an experimental model of breast carcinoma.[40] Although imatinib is initially effective in patients with metastatic GIST, secondary resistance is a great clinical problem.[41] Therefore, development of a novel therapeutic modality is necessary. Further study will be needed to investigate fascin-1 as a potential therapeutic target and the efficacy of migrastatin for patients with metastatic GIST.

In conclusion, we identified *miR-133b* as a possible candidate miRNA downregulated along with the progression of GIST. Overexpression of fascin-1, a possible target of *miR-133b*, was correlated with several aggressive pathological factors and worse prognosis, suggesting that fascin-1 may contribute to the progression of GISTs, and that fascin-1 may be a useful biomarker to predict the aggressive behavior in GIST.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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