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Differential microRNA expression profiles between malignant rhabdoid tumor and epithelioid sarcoma: *miR193a-5p* is suggested to downregulate *SMARCB1* mRNA expression

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Malignant rhabdoid tumor and epithelioid sarcoma are classified as tumors of uncertain differentiation. However, it is controversial whether these tumors are distinct entities because they share similar histological and immunohistochemical features such as the existence of rhabdoid cells or complete loss of SMARCB1 protein expression. MicroRNAs are small non-coding RNAs, and it is suggested that knowledge of microRNA expression profiles in cancer may have substantial value for diagnostics. We first analyzed microRNA expression profiles in 13 frozen materials (five malignant rhabdoid tumors, two proximal type epithelioid sarcomas, and six conventional type epithelioid sarcomas) and subsequently examined the specific microRNA expressions in 29 paraffin-embedded materials (8 malignant rhabdoid tumors, 13 proximal type epithelioid sarcomas, and 8 conventional type epithelioid sarcomas) and 13 previously described frozen materials by quantitative RT–PCR. According to the unsupervised hierarchical clustering of microRNA, proximal type epithelioid sarcoma and conventional type epithelioid sarcoma were classified into the same category, whereas malignant rhabdoid tumor was a distinct category from both types of epithelioid sarcoma. In addition, when malignant rhabdoid tumor with *SMARCB1* gene alterations and proximal type and conventional type epithelioid sarcoma with no *SMARCB1* gene alterations were compared, 56 microRNAs were isolated as being significantly different (ANOVA, $P<0.05$). Among them, quantitative RT–PCR using frozen and paraffin-embedded materials demonstrated that expression levels of *miR193a-5p* ($P=0.002$), which has been suggested to downregulate *SMARCB1* mRNA expression, showed statistically different expression levels between malignant rhabdoid tumor and epithelioid sarcoma with no *SMARCB1* gene alterations. These results suggest that epithelioid sarcoma, especially proximal type epithelioid sarcoma, and malignant rhabdoid tumor are distinct tumors with respect to the microRNA expression profiles and that *miR193a-5p* may have an important role in the inhibition of *SMARCB1* mRNA expression.
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Malignant rhabdoid tumor and epithelioid sarcoma are rare tumors of uncertain differentiation as defined by WHO classification.[1,2] Malignant rhabdoid tumor was originally described in 1978 as a rhabdomyosarcomatoid variant of Wilms' tumor. Malignant rhabdoid tumor arises in varying anatomical locations such as soft tissue, liver, or the central nervous system of infancy or childhood[3,4] while epithelioid sarcoma is a distinctive soft-tissue sarcoma that typically occurs in the distal extremities of adolescents and young adults.[1] In 1997, a large cell variant (proximal type) of epithelioid sarcoma was first described.[1] Proximal type epithelioid sarcoma often arises in the body

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trunk, such as the pelvis, perineum, and genital tract of young to middle-aged adults, and often pursues a rather more aggressive clinical course than the so-called conventional type epithelioid sarcoma.[1,4]

Epithelioid sarcoma, especially proximal type epithelioid sarcoma, shows rhabdoid features and epithelial differentiation demonstrated by immunohistochemical expression of epithelial markers, such as cytokeratin and epithelial membrane antigen, and loss of SMARCB1 protein expression. These features resemble those of malignant rhabdoid tumor.[5,6] Some data exist regarding the histological and immunohistochemical differences between proximal type epithelioid sarcoma and malignant rhabdoid tumor,[7] but such findings are not yet conclusive.

MicroRNAs are small non-coding RNAs of 20–22 nucleotides, typically excised from 60- to 110-nucleotide foldback RNA precursor structures.[8] MicroRNAs exert multiple biological functions by negatively regulating the expression of their target genes involved in development, differentiation, apoptosis, and cell proliferation.[9] It is suggested that knowledge of microRNA expression profiles in cancer may have substantial value for diagnostic and prognostic determinations, as well as for eventual therapeutic intervention, because the microRNA expression profiles potentially reflect the developmental lineage and differentiation state of cancer.[10]

In the present study, we analyzed genome-wide microRNA expressions in malignant rhabdoid tumor and epithelioid sarcoma to test whether these sarcomas are distinct tumor entities and that may have a role in the downregulation of SMARCB1 microRNA expression.

Materials and methods

Patients

Malignant rhabdoid tumor and epithelioid sarcoma in the present study were selected from among more than 17 000 cases of bone and soft-tissue tumors registered in the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan between 1978 and 2012. In all, 42 specimens (malignant rhabdoid tumor, 13 cases; proximal type epithelioid sarcoma, 15 cases; conventional type epithelioid sarcoma, 14 cases) were available. These diagnoses were based on light microscopic examination with hematoxylin and eosin staining according to the most recent WHO classification (malignant rhabdoid tumor and epithelioid sarcoma).[1,2] Moreover, immunoperoxidase procedures using the streptavidin-biotin-peroxidase method were carried out in all cases to confirm the diagnosis. In addition, all cases showed a complete loss of SMARCB1 protein expression, and most cases were previously analyzed for *SMARCB1* gene alterations by our group.[6,11] Each sample was prepared from a different patient.

MicroRNA microarray analysis was performed in 13 frozen samples (malignant rhabdoid tumor, five cases; proximal type epithelioid sarcoma, two cases; conventional type epithelioid sarcoma, six cases) (Figure 1 and Table 1) and 3 frozen samples of surrounding non-tumorous skeletal muscle that were collected from patients with various types of sarcoma as controls, and quantitative RT–PCR analysis was carried out in all 42 samples: 29 paraffin-embedded materials (8 malignant rhabdoid tumors, 13 proximal type epithelioid sarcomas and 8 conventional-type epithelioid sarcomas) and the 13 frozen samples described above. *SMARCB1* gene alteration analyses were not available in three paraffin-embedded malignant rhabdoid tumor cases because of the small amount of material, whereas the remaining nine malignant rhabdoid tumor cases demonstrated *SMARCB1* gene alteration causing loss of *SMARCB1* protein expression. Two cases of paraffin-embedded proximal type epithelioid sarcoma had *SMARCB1* gene alteration causing the loss of SMARCB1 protein expression (Table 2), but the remaining 23 epithelioid sarcoma cases showed no such *SMARCB1* gene alterations.

MicroRNA Microarray

Total RNAs, including microRNAs, were extracted from frozen and paraffin-embedded samples using the miRNeasy Mini Kit (Qiagen in Japan, Tokyo, Japan) according to the manufacturer's instructions.

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 837 anti-sense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA database (http://microrna.sanger.ac.uk/sequences/). The chips were carefully washed, and fluorescent signals were scanned with the ScanArray Lite Scanner (PerkinElmer, Waltham, MA) and analyzed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA). The raw data of each spot were normalized by substitution with the 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 microRNA 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.

Quantitative RT–PCR

RNAs isolated from frozen or paraffin-embedded materials were reverse transcribed and PCR amplified

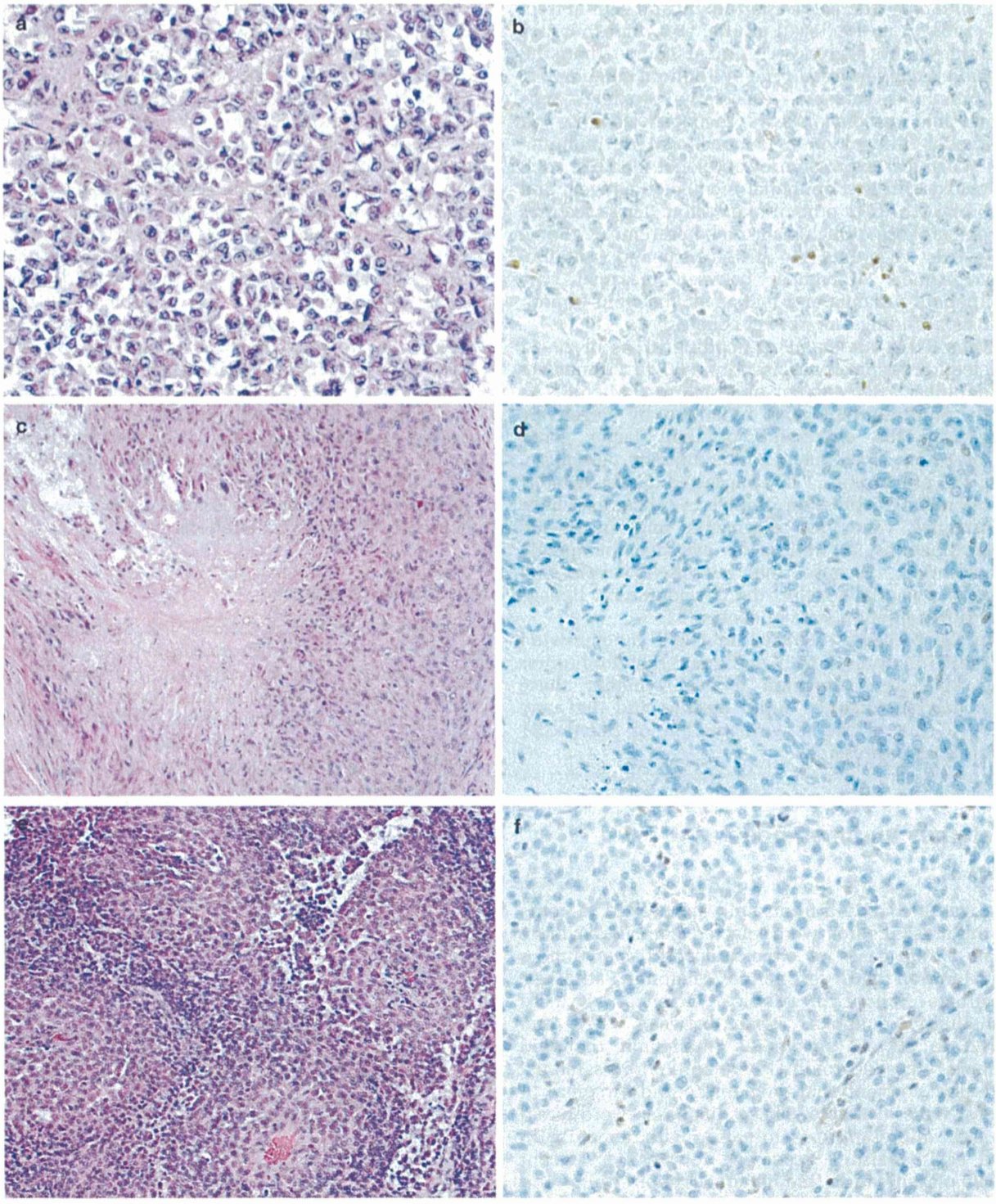

Figure 1 Hematoxylin and eosin histologic features and SMARCB1 immunoreactivities in malignant rhabdoid tumor and epithelioid sarcoma cases. (**a** and **b**) Malignant rhabdoid tumor case (MRT-5 at Table 1). (**c** and **d**) Conventional type epithelioid sarcoma case (CES-4 in Table 1). (**e** and **f**) Proximal type epithelioid sarcoma case (PES-3 in Table 1). Immunohistochemically, in the cases with loss of SMARCB1 protein expression, no nuclear expression is observed in tumor cells, whereas infiltrating lymphocytes or vascular endothelial cells show immunoreactivity (**b**, **d** and **f**).

Table 1 Profiles of frozen samples using microRNA microarray analysis

Diagnosis	*Age*	*Sex*	*Site*	*Prognosis*	*SMARCB1 immunoreactivity*	*SMARCB1 gene alteration*
MRT[a]-3	0 years 5 months	F	Neck	12 DOD[b]	No expression	Homozygous deletion (exon 1–9)
MRT-4	0 years 0 months 0 days	F	Retroperitoneum	2 DOD	No expression	Nonsense mutation (exon 4, codon 128)
MRT-5	2 years	M	rt. kidney	82 NED[c]	No expression	Homozygous deletion (exon 1–6)
MRT-6	0 years 5 months	M	rt. kidney	75 NED	No expression	Homozygous deletion (exon 1–9)
MRT-7	0 years 4months	M	Liver	12 DOD	No expression	Homozygous deletion (exon 1–9)
PES[d]-2	33 years	M	lt. buttock	54 NED	No expression	No alteration
PES-3	40 years	M	rt. groin	47 NED	No expression	No alteration
CES[e]-1	71 years	F	rt. thigh	64 NED	No expression	No alteration
CES-2	34 years	M	rt. forearm	166 NED	No expression	No alteration
CES-3	62 years	F	rt. forearm	115 NED	No expression	No alteration
CES-4	22 years	F	rt. forearm	71 NED	No expression	No alteration
CES-5	58 years	M	lt. hand	37 AWD[f]	No expression	No alteration
CES-6	33 years	M	lt. hand	10 DOD	No expression	No alteration

[a]Malignant rhabdoid tumor.
[b]Dead of disease.
[c]No evidence of disease.
[d]Proximal type epithelioid sarcoma.
[e]Conventional type epithelioid sarcoma.
[f]Alive with disease.

Table 2 MicroRNA expression values of proximal type epithelioid sarcoma with *SMARCB1* gene alteration and these mean values of malignant rhabdoid tumor, proximal type epitheloid sarcoma, and conventional type epithelioid sarcoma

	miR-19a/SNORD25	*miR-193a-5p/SNORD25*
Two proximal type epithelioid sarcoma with SMARCB1 gene alteration cases		
Case 3, Table 4[a] (2 bp deletion, exon 3)	17.8	4.8
Case 5, Table 4[a] (1 bp deletion, exon 9)	18.3	6.1
Mean value of malignant rhabdoid tumor	10.2	3.1
Mean value of proximal type epithelioid sarcoma	12.1	23.7
Mean value of conventional type epithelioid sarcoma	4.7	14.6

[a]Previously reported cases in our article.[6]

with the miScript II RT kit and miScript SYBR PCR kit (Qiagen, Valencia, CA, USA) using an ABI Prism 7500 Sequence Detection system (Applied Biosystems) following the manufacturer's protocols. Quantitations for *miR-19a* and *miR193a-5p* were performed using predeveloped miScript reagents (*miR-19a*, MS00003192; *miR193a-5p*, MS00008932). A small non-coding RNA gene (*SNORD25*, MS00014007) was used for normalization. Relative expression levels were calculated following the comparative threshold cycle method: final numerical value = *miR-19a* or *miR193a-5P* value/*SNORD25* value.

Results

MicroRNA Expression Profiles in Malignant Rhabdoid Tumor and Epithelioid Sarcoma

An unsupervised hierarchical clustering based on the relative expression of microRNAs with valid duplicate spots showed distinctive expression profiles for each sample type, and non-tumorous skeletal muscle, malignant rhabdoid tumor, and epithelioid sarcoma groups examined were properly clustered into the specific tissue type category. However, the microRNA profiles in the proximal type epithelioid sarcoma group and conventional type epithelioid sarcoma group were intermingled (Figure 2).

To identify the microRNAs that were differentially expressed among malignant rhabdoid tumor, proximal type epithelioid sarcoma and conventional type epithelioid sarcoma, we compared the expression levels of microRNAs among the three groups using ANOVA ($P<0.05$). As shown in Figure 3, 56 microRNAs were differentially expressed. Three of these 56 microRNAs were identified as microRNAs with the potential of *SMARCB1* mRNA inhibition according to the MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5): namely *miR-19a*, *miR-193a-5p*, and *miR-486-5p*. However, the possibility of a function with *INI1* mRNA inhibition was unlikely for *miR-486-5p* because the mean expression value of miR-486-5p was much higher in non-tumorous skeletal muscle (507.5) than in malignant rhabdoid tumor (32.7), conventional type epithelioid sarcoma

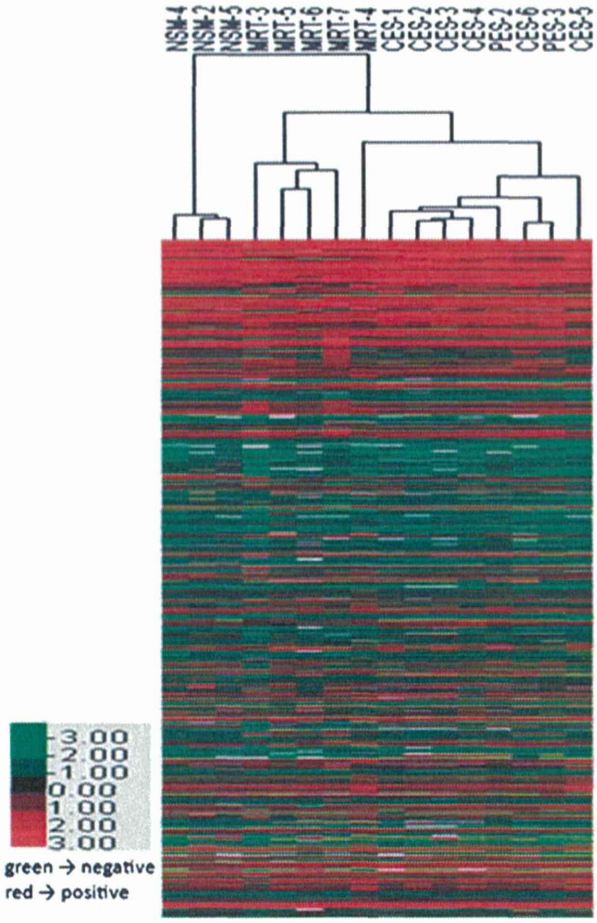

Figure 2 Global microRNA expression and unsupervised hierarchical clustering of malignant rhabdoid tumor (MRT), proximal type epithelioid sarcoma (PES), conventional type epithelioid sarcoma (CES), and non-tumorous skeletal muscle (NSM).

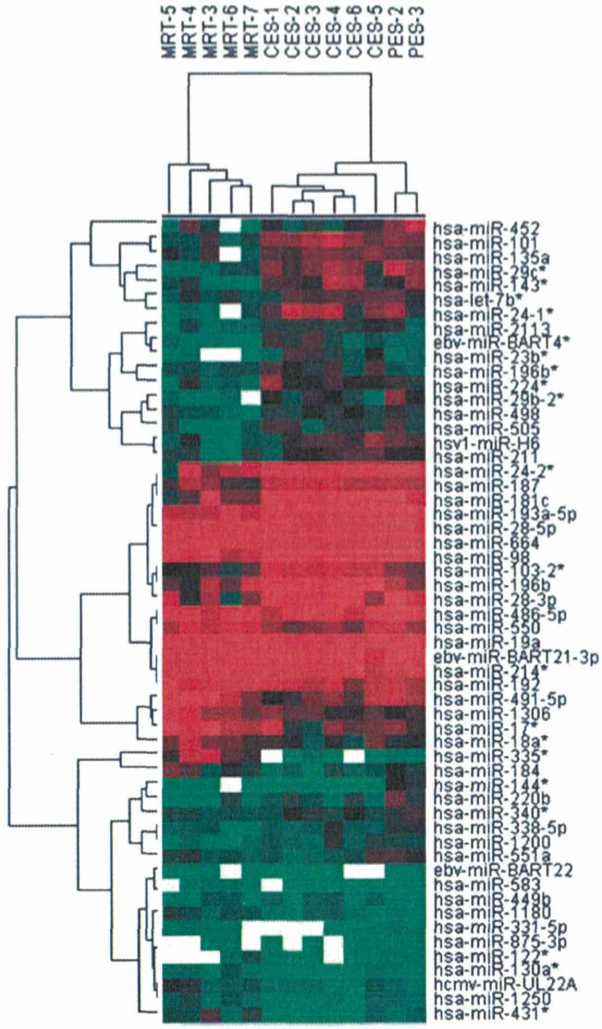

Figure 3 Fifty-six microRNAs showing significant differences between malignant rhabdoid tumor (MRT), proximal type epithelioid sarcoma (PES) and conventional type epithelioid sarcoma (CES) by ANOVA ($P<0.05$).

(27.3), or proximal type epithelioid sarcoma (59.3) (Table 3).

MiR-19a and miR-193a-5p Expressions

The results of microRNA expressions are summarized in Table 2 and Figure 4. The mean *MiR-19a* expression values of malignant rhabdoid tumor, proximal type epithelioid sarcoma with no *SMARCB1* gene alterations, and conventional type epithelioid sarcoma with no *SMARCB1* gene alterations were 10.2, 12.1, and 4.7, respectively (Figure 4a). However, none of the differences were statistically significant (malignant rhabdoid tumor *vs* proximal type epithelioid sarcoma *vs* conventional type epithelioid sarcoma, $P=0.09$; malignant rhabdoid tumor *vs* proximal type epithelioid sarcoma, $P=0.65$; malignant rhabdoid tumor *vs* conventional type epithelioid sarcoma, $P=0.12$; proximal type epithelioid sarcoma *vs* conventional type epithelioid sarcoma, $P=0.13$). The mean *MiR-193a-5p* expression values of malignant rhabdoid tumor, proximal type epithelioid sarcoma with no *SMARCB1* gene alterations, and conventional type epithelioid sarcoma with no *SMARCB1* gene alterations were 3.1, 23.7, and 14.6, respectively (Figure 4b). The mean *MiR-193a-5p* expression values in epithelioid sarcoma with no *SMARCB1* gene alterations, both proximal type epithelioid sarcoma and conventional type epithelioid sarcoma, were significantly higher than that in malignant rhabdoid tumor (malignant rhabdoid tumor *vs* proximal type epithelioid sarcoma *vs* conventional type epithelioid sarcoma, $P=0.002$; malignant rhabdoid tumor *vs* proximal type epithelioid sarcoma, $P=0.002$; malignant rhabdoid tumor *vs* conventional

Table 3 MicroRNA expression ratios of non-tumorous skeletal muscle, malignant rhabdoid tumor, conventional type epitheloid sarcoma, and proximal type epithelioid sarcoma in microarray analysis

	NSM[a]*-2*	*NSM-4*	*NSM-5*	*MRT*[b]*-3*	*MRT-4*	*MRT-5*	*MRT-6*	*MRT-7*	*CES*[c]*-1*	*CES-2*	*CES-3*	*CES-4*	*CES-5*	*CES-6*	*PES*[d]*-2*	*PES-3*
miR-486-5p	529.1	475.6	517.9	17.9	52.8	27.4	31.6	33.9	16.4	23.2	31.7	28.1	37.1	18.2	63.7	54.8
	Mean; 507.5			Mean; 32.7					Mean; 27.3						Mean; 59.3	
miR-19a	106.7	61.4	36.1	261.5	199.9	214.6	163.5	187.8	125.4	41.4	64.9	63.8	54.9	65.9	87.1	52.5
	Mean; 68.1			Mean; 205.5					Mean; 70.1						Mean; 69.8	
miR-193a-5p	14.5	14.6	12.1	16.9	16.5	17.2	29.8	18.4	42.8	50.9	51.1	47.9	52.9	38.1	52.9	35.0
	Mean; 13.7			Mean; 19.8					Mean; 49.1						Mean; 44	

[a]Non-tumorous skeletal muscle.
[b]Malignant rhabdoid tumor.
[c]Conventional type epithelioid sarcoma.
[d]Proximal type epithelioid sarcoma.

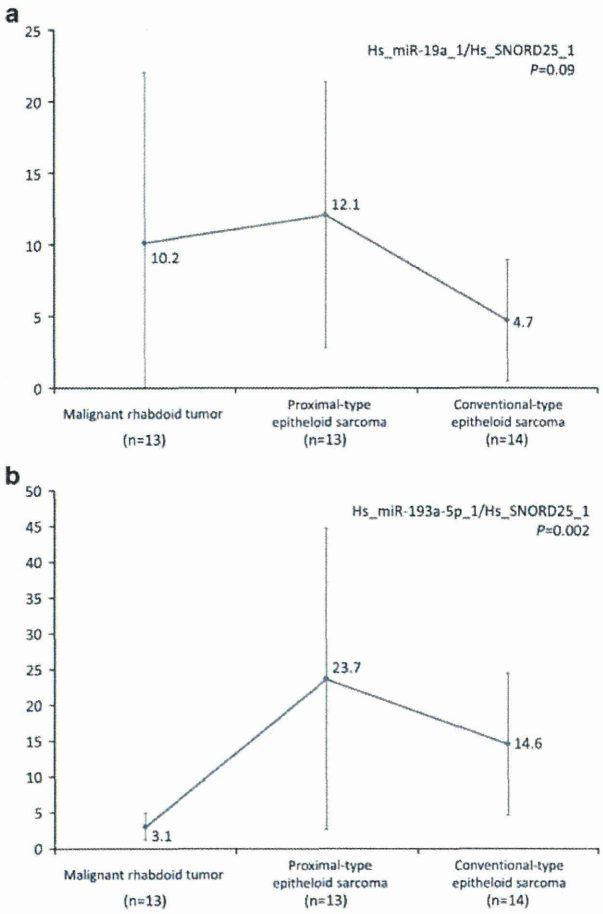

Figure 4 The expression values of *miR-19a* (**a**) and *miR193a-5p* (**b**) in malignant rhabdoid tumor, proximal type epithelioid sarcoma with no SMARCB1 gene alterations, and conventional type epithelioid sarcoma with no SMARCB1 gene alterations. There were statistically significant differences among the three groups with respect to expression levels of *miR193a-5p* ($P=0.002$) but not *miR-19a* ($P=0.09$).

type epithelioid sarcoma, $P=0.0004$; proximal type epithelioid sarcoma *vs* conventional type epithelioid sarcoma, $P=0.16$).

The microRNA expression values of the two proximal type epithelioid sarcoma samples with *SMARCB1* gene alteration are also exhibited in Table 2. In the two proximal type epithelioid sarcoma samples with *SMARCB1* gene alteration, the *MiR-193a-5p* expression values (4.8, 6.1) were closer to that of malignant rhabdoid tumor (3.1) than to that of the proximal type epithelioid sarcoma samples with no *SMARCB1* gene alterations (23.7).

Discussion

The histological features of malignant rhabdoid tumor particularly resemble proximal type epithelioid sarcoma rather than conventional type epithelioid sarcoma; however, several factors distinguishing malignant rhabdoid tumor from epithelioid sarcoma have been reported.[4] Clinically, the peak age for the incidence of proximal type epithelioid sarcoma is early adulthood and adolescence; patients younger than 10 years are rare. In contrast, malignant rhabdoid tumor usually occurs in infancy or childhood. Furthermore, the biological behavior of proximal type epithelioid sarcoma is less aggressive than that of malignant rhabdoid tumor. Immunohistochemically, approximately half of all epithelioid sarcoma cases are positive for CD34 and dysadherin, whereas malignant rhabdoid tumor cases reveal no immunoreactivities for these markers.[12] By contrast, glypican-3 immunoexpression is found in about half of malignant rhabdoid tumor cases.[7] However, microRNA expression profiles in malignant rhabdoid tumor and epithelioid sarcoma have never been investigated.

In terms of microRNA expression profiles of our current study, malignant rhabdoid rumor and epithelioid sarcoma were significantly different, but no significant difference was found between proximal type and conventional type epithelioid sarcoma. Histologically, conventional type epithelioid sarcoma shows a characteristic pseudogranulomatous pattern and a proliferation of eosinophilic epithelioid and spindle-shaped cells exhibiting slight nuclear atypia, vesicular nuclei, and small nucleoli with central necrosis,[1] whereas proximal type epithelioid sarcoma shows a multinodular growth pattern and consists of large

epithelioid carcinoma-like cells having marked cytological atypia, vesicular nuclei, and prominent nucleoli; these two histologies have obvious differences.[1] However, our current results for microRNA expression profiles support the present WHO classification in which conventional type and proximal type epithelioid sarcoma are in the same category, whereas malignant rhabdoid tumor and epithelioid sarcoma are different.[1,2]

SMARCB1 is one of the evolutionarily conserved core subunits in the ATP-dependent SWI/SNF complex and is ubiquitously expressed in the nuclei of all normal cells.[13,14] Complete loss of SMARCB1 immunoexpression has been detected in all malignant rhabdoid tumor cases except in rare examples and in almost all epithelioid sarcoma cases.[5,7] About 20% of malignant rhabdoid tumor cases with complete loss of SMARCB1 protein expression have no alterations sufficient to suppress the expression of gene products at either the DNA or RNA level.[15,16] Meanwhile, in proximal type epithelioid sarcoma, there were discrepancies in the frequency of *SMARCB1* gene alteration between the investigations of Modena *et al* (100%), that of Papp *et al* (19%: rate of biallelic alteration explaining the loss of SMARCB1 function in both types of epithelioid sarcoma), that of Sullivan *et al* (100%), and that of our previous report (25%).[6,17–19] Whatever the causes, no mechanism for the inactivation of the *SMARCB1* gene product in malignant rhabdoid tumor and epithelioid sarcoma cases with no gene alteration has ever been clarified, and the possibility of regulation by specific microRNA and/or more factors has been considered likely.

The results of our present study suggest that *miR-193a-5p* can have the role of negatively regulating *SMARCB1* mRNA because the mean *mi-193a-5p* expression value of microarray analysis in epithelioid sarcomas with no *SMARCB1* gene alterations (conventional-type; 49.1, proximal type; 44.0) was higher than that in non-tumorous skeletal muscle (13.7), and the *miR-193a-5p* expression value in epithelioid sarcoma with no *SMARCB1* gene alterations was significantly higher than that in malignant rhabdoid tumor (malignant rhabdoid tumor *vs* proximal type epithelioid sarcoma, $P=0.002$; malignant rhabdoid tumor *vs* conventional type epithelioid sarcoma, $P=0.0004$; proximal type epithelioid sarcoma *vs* conventional type epithelioid sarcoma, $P=0.16$). In addition, the *miR193a-5p* expression values of two cases of proximal type epithelioid sarcoma with *SMARCB1* gene alteration (4.8, 6.1) were closer to that of malignant rhabdoid tumor (3.1) than those of epithelioid sarcoma with no *SMARCB1* gene alteration (proximal type epithelioid sarcoma, 23.7; conventional type epithelioid sarcoma, 14.6). The function of *miR-193a-5p* in *SMARCB1* mRNA inhibition has not yet been reported. However, it was previously reported that *miR-193a-5p* might be involved in the mechanism of a predictive tool for ifosfamide response in osteosarcoma or as a target of the YY1-APC regulatory axis in human endometrioid endometrial adenocarcinoma.[20,21] The present study concentrated on analysis in clinical samples, and we did not conduct a verification test using cell lines. Therefore, we cannot confirm absolutely the role of *miR-193a-5p*.

In summary, we analyzed microRNA expressions in malignant rhabdoid tumor and epithelioid sarcoma. In microarray analysis, conventional type and proximal type epithelioid sarcoma showed almost the same microRNA expression profiles, but both types of epithelioid sarcoma had different profiles from malignant rhabdoid tumor. Accordingly, epithelioid sarcoma, especially proximal type epithelioid sarcoma, and malignant rhabdoid tumor are suggested to be distinct tumors, and both types of epithelioid sarcoma are suggested to be involved in the same category. In addition, the miR193a-5p expression value in malignant rhabdoid tumor was significantly higher than those in proximal type and conventional type epithelioid sarcoma with no *SMARCB1* gene alterations ($P=0.002$). Therefore, it is suggested that *miR193a-5p* may have the potential to inhibit *SMARCB1* mRNA.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Original contribution

Close correlation between CXCR4 and VEGF expression and frequent CXCR7 expression in rhabdomyosarcoma☆,☆☆

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Summary CXC chemokine receptor 4 (CXCR4) expression is reportedly correlated with both vascular endothelial growth factor (VEGF) expression and poor prognosis in a variety of cancers. Its relation to CXC chemokine receptor 7 (CXCR7) is also noted in several malignancies, including rhabdomyosarcoma (RMS) cell lines. However, the correlations between these chemokine receptors and angiogenic factors have not yet been adequately investigated in RMS clinical specimens. By immunohistochemistry, we assessed CXCR4, CXCR7, CC chemokine receptor 6, CC chemokine receptor 7, VEGF expression, microvessel density, and MIB-1 labeling index in 82 formalin-fixed RMS specimens, including 34 primary alveolar RMS and 44 primary embryonal RMS (ERMS). Twenty-six frozen samples were available for investigation by quantitative reverse transcription polymerase chain reaction to detect the messenger RNA expression levels of these molecules. We also evaluated their significance with respect to clinicopathological factors and patient survival rates. Primary RMS showed high expression of CXCR7 (83.1%) regardless of the histologic subtype. High cytoplasmic CXCR4 and high VEGF expression revealed significant correlations in both ERMS and alveolar RMS ($P = .0051$ and $P = .0003$, respectively). By univariate analysis of ERMS cases, the tumors with high VEGF expression showed significantly poor prognoses ($P = .0017$). High VEGF expression also was the independent adverse prognostic factor for ERMS. Because CXCR4, CXCR7, and VEGF are widely expressed in RMS, the combination of these antagonists may provide a potential target for molecular therapy.

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1. Introduction

Rhabdomyosarcoma (RMS) is the most common malignant soft tissue sarcoma in childhood and adolescence. There are 2 major histologic subtypes in RMS: alveolar RMS (ARMS) and embryonal RMS (ERMS). The former is well known to be associated with *PAX3-FKHR* or *PAX7-FKHR* gene fusions and with significantly poor prognosis. Meanwhile, no specific fusion gene has been identified in ERMS [1].

Many kinds of chemokines display various roles in immunity, regulating angiogenesis, promoting the proliferation of tumor cells, and mediating organ-specific metastases [2]. Chemokine receptors, especially CXC chemokine receptor 4 (CXCR4) [3-7] and CXC chemokine receptor 7 (CXCR7) [8,9], have been suggested to play an important role in metastatic behavior to specific target organs [10]. CC chemokine receptor 7 (CCR7) is primarily responsible for lymph node metastases [11,12], whereas CC chemokine receptor 6 (CCR6) is suggested to have relation to liver metastases [13] and tumor progression [14].

Vascular endothelial growth factor (VEGF), known as a critical mediator of angiogenesis and tumor proliferation, has revealed its overexpressed status in a variety of cancers [15]. Thus, the therapeutic approaches targeting VEGF have been explored in several cancers [16-18].

CXCR4 has been believed to be the only receptor that binds stromal cell–derived factor (SDF-1). Recently, a new SDF-1 binding receptor, CXCR7, was identified through an experiment that revealed discrepancies between CXCR4 expression and SDF-1 binding on different cell lines, using cells from CXCR4-deficient mice [19].

In RMS cell lines, it has been reported that CXCR4 was expressed at much higher levels by highly metastatic ARMS lines, whereas CXCR7 was expressed in both ARMS and ERMS, with higher expression in ERMS cell lines [20].

A significant correlation between the messenger RNA (mRNA) levels of VEGF and CXCR4 in breast cancer tumor tissue was reported in a preliminary investigation [21]. In addition, the autocrine manner of VEGF's involvement in the CXCR4/SDF-1 pathway in the invasion of breast carcinoma cells has been demonstrated [8]. Furthermore, the CXCR4/SDF-1 system was found to be involved with the PI3K/Akt pathway in a breast cancer cell line [21] and with the ERK pathway in a pancreatic cancer cell line [22].

In the present study, we immunohistochemically evaluated CXCR4, CXCR7, CCR6, CCR7, and VEGF protein expression to investigate these protein expressions in a large series of RMS clinical cases and examined the mRNA expression levels of *CXCR4*, *CXCR7*, *CCR6*, *CCR7*, and *VEGF* in frozen samples using quantitative reverse transcription polymerase chain reaction (RT-PCR). Moreover, we compared these results with clinicopathological parameters, angiogenesis factors, and prognosis in RMS.

There have been no reports on the relationship of metastasis-related chemokine receptors and angiogenesis factors in the aspects of tissue expression in large series of clinical RMS cases. The investigation for these protein expressions in the clinical tumor tissue could aid in the search for new potential therapeutic targets.

2. Materials and methods

2.1. Patients and tissue specimens

Eighty-two paraffin-embedded RMS specimens obtained from 78 patients were collected from the soft tissue tumor file registered between 1976 and 2007 at the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. The 82 specimens from these 78 patients included 78 primary tumors, 3 metastatic tumors (2 in lung and 1 in thigh), and 1 locally recurrent tumor. The histologic diagnosis of RMS and its subtype was confirmed according to the latest World Health Organization classification [1]. The anatomical locations of the primary tumor sites were categorized as favorable or unfavorable tumor sites according to Intergroup Rhabdomyosarcoma Study V (IRS-V) [23]. The patients were classified according to the pretreatment staging system by Intergroup Rhabdomyosarcoma Study Group [23]. Furthermore, 26 of the specimens were also snap frozen in liquid nitrogen at the time of the surgical procedure and stored at −80°C until use. The institutional review board at Kyushu University approved this study (permission code: 25-143).

2.2. Immunohistochemistry

Immunohistochemistry was performed in 82 tumors, using formalin-fixed tissue sections in concordance with frozen material. Sections were cut at widths of 4 μm from paraffin-embedded material, dewaxed with xylene, and rehydrated through a graded series of ethanol. After the inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted.

The following antibodies were used as primary antibody: anti-CXCR4 (12G5, monoclonal, 1:100; BD PharMingen, San Diego, CA), anti-CXCR7 (polyclonal, 1:200; GeneTex, Irvine, CA), anti-CCR6 (11A9, monoclonal, 1:200; BD Pharmingen), anti-CCR7 (polyclonal, 1:250; Capralogics, Hardwick, MA), anti-VEGF (A-20, polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD31 (JC70A, monoclonal, 1:20; Dako, Glostrup, Denmark), and anti–Ki-67 (MIB-1, monoclonal, 1:100; DAKO). For staining with CXCR4, CXCR7, CCR6, CCR7, VEGF, and Ki-67, sections were pretreated with microwave irradiation in citrate buffer or EDTA buffer for antigen retrieval. As for CD31, sections were pretreated with trypsin for 30 minutes.

Sections from human tonsils for CXCR4, CCR6, and CCR7 and sections from human renal cell carcinoma for CXCR7 were used as positive controls [24,25].