Tohoku J. Exp. Med., 2019, **247**, 119-127

Renal Epithelioid Angiomyolipoma Undergoing Aggressive Clinical Outcome: The MDM2 Expression in Tumor Cells of Two Cases

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Epithelioid angiomyolipoma (EAML) has been known as a potentially malignant tumor which occasionally recur and/or metastasize to other organs, and clinically and pathologically recognized as distinct entity. However, the mechanisms of recurrence and/or metastasis (recurrence/metastasis) has still remained unknown. Here, we report two cases of renal EAML associated with recurrence/metastasis, and three cases of EAML in kidney or liver without recurrence/metastasis. According to the previous histological predictive models of EAML, the primary tumor was classified as low risk group in one of the cases with recurrence/metastasis in spite of its malignant behavior. Therefore, we considered that further investigation about the mechanisms of recurrence/metastasis in EAML is required for a malignancy prediction. We focused on some cell-cycle modulators, including mouse double minute 2 homolog (MDM2), which is ubiquitin ligase well-known to promote malignant behaviors by p53 ubiquitination and degradation, and also other cellular processes including genomic instability and epithelial-mesenchymal transition in p53-independent manners in various human malignancies. Immunohistochemical evaluation revealed that MDM2 protein expression increased stepwise throughout every steps of metastasis/recurrence in both cases, although it was negative in primary tumors. In conclusion, this is the first study demonstrating that MDM2 could play an important role in the molecular mechanisms of recurrence/metastasis of EAML. Further analyses focusing on MDM2 pathway could contribute to the identification of novel prognostic factors and/or therapeutic targets in EAML patients.

Keywords: epithelioid angiomyolipoma; immunohistochemistry; MDM2; metastasis; recurrence
Tohoku J. Exp. Med., 2019 February, **247** (2), 119-127.

Introduction

Angiomyolipoma (AML) is a mesenchymal tumor composed of variable ratios of adipose tissue and smooth muscle cells, and also characterized by abnormal thick-walled blood vessels. AML belongs to perivascular epithelioid cell tumors (PEComas), harboring the proliferation of perivascular epithelioid cells (Moch et al. 2016). Epithelioid angiomyolipoma (EAML) is a rare variant of AML and defined as that AML consists of epithelioid cells accounting for at least 80% of the whole tumor. AML is generally considered a benign tumor but some EAML cases have been reported to undergo malignant behavior, such as recurrence and metastases. Therefore, it is considered that EAML should be recognized as distinct entity clinically and pathologically (Mete and van der Kwast 2011; Varma et al. 2011).

Risk factors of progression of EAML were reported by Brimo et al. (2010) and Nese et al. (2011). Nese et al. (2011) studied the clinicopathologic parameters in 41 cases with pure EAML arising in kidney. They proposed the parameters such as the presence of tuberous sclerosis complex or concurrent AML, necrosis, tumor size, external extension and/or renal vein involvement, and carcinoma-like growth pattern as potential factors predicting recurrence, metastasis, or even demise of the patients. Brimo et al. (2010) also examined 40 cases with renal AML harboring atypical epithelioid components, and proposed a predictive model for malignant behavior which consist of 4 atypical features. As described above, the histological

Received December 11, 2018; revised and accepted February 5, 2019. Published online February 22, 2019; doi: 10.1620/tjem.247.119.
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characters to predict malignancy were examined so far, however, exact molecular mechanisms of aggressive biological behaviors in EAML have been remained unknown. Elucidating the mechanisms may help to find out new risk factors of progression and therapeutic targets.

Therefore, we focused on molecules known to modulate cell cycles, such as MDM2, p16, CDK4 and others, to explore the potential correlation of increased cell proliferation with aggressive biological behavior of the tumor. Especially, MDM2 is an E3 ubiquitin ligase that is well known to ubiquitinate p53 for proteasomal degradation and promote cell cycle (Bohlman and Manfredi 2014). In addition, MDM2 has been reported to promote other cellular processes including genomic instability and epithelial-mesenchymal transition in p53-independent manners in various human malignancies. We compared the changes of the expression levels of these molecules between primary and metastatic lesions in the same patients.

Materials and Methods

Immunohistochemistry

The antibodies used for the immunohistochemical analysis, their catalogue number, clones, locations in cells, host, dilution, antigen retrieval methods, tissues used for positive control, and sources are summarized in Table 1. Antigen retrieval using microwave was performed by heating the slides in citric acid for 15 min in a microwave. Antigen retrieval using autoclave was performed by autoclaving the slides in citric acid (pH 6.0) or in Instant Antigen Retrieval Solution H (LSI Medience, Corp., Tokyo, Japan) (pH 7.0) for 5 min at 121°C. We immunostained the sections with Histofine Kit (Nichirei bioscience, Tokyo, Japan) for all antibodies excluding GLUT1, according to the manufacturer's instructions. Envision (Dako Japan, Kyoto, Japan) was used for immunostaining of GLUT1. Immune complexes were detected with 3,3′-diaminobenzidine (DAB), and counterstained with hematoxylin. The MDM2 antibody used in

Table 1. Antibodies used for immunohistochemical analysis.

Antibody	Catalog	Clone	Location	Host	Dilution	Antigen retrieval	Control	Source
MDM2	ab3110	SMP 14	N	M	1:5000	autoclave (pH 7.0)	colon cancer	Abcam
β-catenin	610153	14/Beta-Catenin	N	M	1:200	microwave	breast cancer	BD Biosciences
GATA3	ACR405	L50-823	N	M	1:500	autoclave (pH6.0)	breast cancer	Biocare Medical
p16	554079	G175-1239	N	M	1:100	autoclave (pH6.0)	cervical cancer	BD Biosciences
cyclin D1	413521	SP4	N	R	1:1	autoclave (pH 7.0)	breast cancer	Nichirei Biosciences
CDK4	sc-260	C-22	N	R	1:200	microwave	liposarcoma	Santa Cruz Biotechnology
c-kit	A4502	polyclonal	M	R	1:100	autoclave (pH6.0)	GIST	DAKO
EGFR	423701	31G7	M	M	1:1	protease	colon cancer	Nichirei Biosciences
GLUT1	07-1401	polyclonal	M	R	1:300	autoclave (pH6.0)	malignant mesothelioma	Millipore
HER2	A0485	polyclonal	M	R	1:1000	autoclave (pH6.0)	breast cancer	DAKO
ER	NCL-L-ER-6F11	6F11	N	M	1:50	autoclave (pH6.0)	uterus	Leica Biosystems Newcastle
PgR	ab51896	1A6	N	M	1:50	autoclave (pH6.0)	breast cancer	Abcam
Bcl-2	M0887	124	C	M	1:80	autoclave (pH6.0)	tonsil	DAKO
Ki-67	M7240	MIB-1	N	M	1:100	autoclave (pH6.0)	tonsil	DAKO
p53	NCL-L-p53-DO7	DO-7	N	M	1:200	microwave	colon cancer	Leica Biosystems Newcastle

Location: C, Cytoplasm; M, Membrane; N, Nucleus.
Host: M, Mouse; R, Rabbit.
Control: GIST, gastrointestinal stromal tumor.
Source: Abcam, Cambridge, UK/ BD Biosciences, San Diego, CA, USA/ Biocare Medical, Concord, CA/ Nichirei Biosciences, Inc., Tokyo, Japan/ Santa Cruz Biotechnology, Santa Cruz, CA, USA/ DAKO, Carpinteria, CA, USA/ Millipore, Billerica, MA, USA/ Leica Biosystems Newcastle Ltd, Newcastle, UK.

this analysis was commonly used in previous reports (Zhang et al. 2015; Li et al. 2016). Ki-67 labelling index (LI) was determined by counting 1,000 tumor cells in the hot spots under × 400 magnification, and calculating the percentage of positively stained nuclei. Not all specimens used for immunohistochemistry were serial sections.

Fluorescence in situ hybridization (FISH)

Three tumors immunopositive for MDM2 were tested by FISH labeling the targeted DNA to examine gene amplification. Slides were hybridized with probes to MDM2 and chromosome 12 centromere (CEP12) using Vysis LSI MDM2 Spectrum Orange Probe and Vysis DNA FISH probe CEP12 (Abott Molecular/Vysis, Des Plaines, IL, USA) according to the manufacturer's instructions. Sections were counterstained with 4,6-diamidino-2-phenylindole and were visualized with a fluorescent microscope. We calculated the ratio of the average number of MDM2 gene and CEP12 gene signals counting randomly 20 tumor cells. We defined an amplification of the MDM2 gene region by a FISH score of 2.0 or above according to the previous report (Asch-Kendrick et al. 2016).

Statistical analysis

All statistical analyses were performed using JMP Pro 14.0.0 (SAS Institute, Japan, Tokyo). Statistical significance was defined as $P < 0.05$ in this study.

Clinical Summary

Two EAML cases with recurrence and/or metastasis (recurrence/metastasis)

Case 1

A 61 years-old male underwent left nephrectomy due to the kidney tumor. At three years after the nephrectomy, three tumors were detected in the liver on a follow-up computed tomography (CT) scan (Fig. 1a). Extended posterior segmentectomy and partial resection of segment 3 was subsequently performed. In three months after this surgery, CT scan revealed a nodule in the left lung and the partial resection of the left lung was performed. All of these tumors were diagnosed as EAML. No genetic analyses were performed.

Case 2

A 36-years-old man underwent right nephrectomy for the kidney tumor. This primary lesion of this case had been previously reported by Konosu-Fukaya et al. (2014). At 6 years after the nephrectomy, a nodule was detected in the post mediastinum on the follow-up fluoro-D-glucose integrated with computed tomography (18F-FDG PET/CT) (Fig. 1b). This mediastinal nodule was surgically resected. Both of these tumors were histologically diagnosed as EAML. No genetic analyses were performed.

Three EAML cases without metastasis/recurrence:

Case 3

An 82 years-old man underwent hepatic left lateral segmentectomy due to the liver tumor measuring 17 mm, suspected as hepatocellular carcinoma. The tumor was pathologically diagnosed as EAML.

Case 4

A 61 years-old woman underwent partial nephrectomy due to the kidney tumor measuring 15 mm, suspected as renal cell clear cell carcinoma. The tumor was pathologically diagnosed as EAML.

Case 5

A 44 years-old man underwent right nephrectomy due to the tumor, which detected as a mass measuring 3 cm on CT 5 years, and grown up a mass of 14 cm. The tumor was pathologically diagnosed as EAML.

Pathological Findings

Case 1

Macroscopically, all primary and recurrent/metastatic lesions demonstrated well-circumscribed nodules measuring up to 8 cm, 5.5 cm and 1 cm in greatest diameter in the kidney (primary site), liver (the first metastatic site) (Fig. 2a) and lung (the second metastatic site) respectively. Histologically, all of these tumors demonstrated monotonous appearance (Fig. 2b, c) and consisted of spindle cells

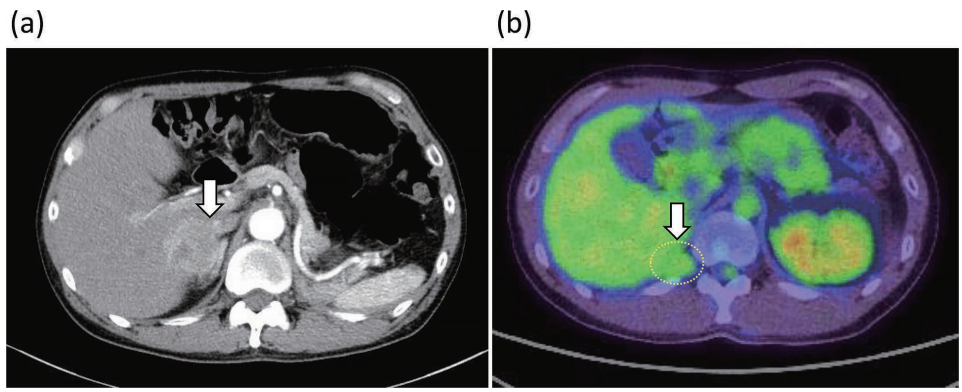

Fig. 1. Computed tomography (CT) scan images of Case 1 and 2.
(a) CT scan showing a mass (arrow) in the liver of Case 1.
(b) Fluoro-D-glucose integrated with CT (18F-FDG PET/CT) showing a nodule (arrow and circle) in the posterior mediastinum of Case 2.

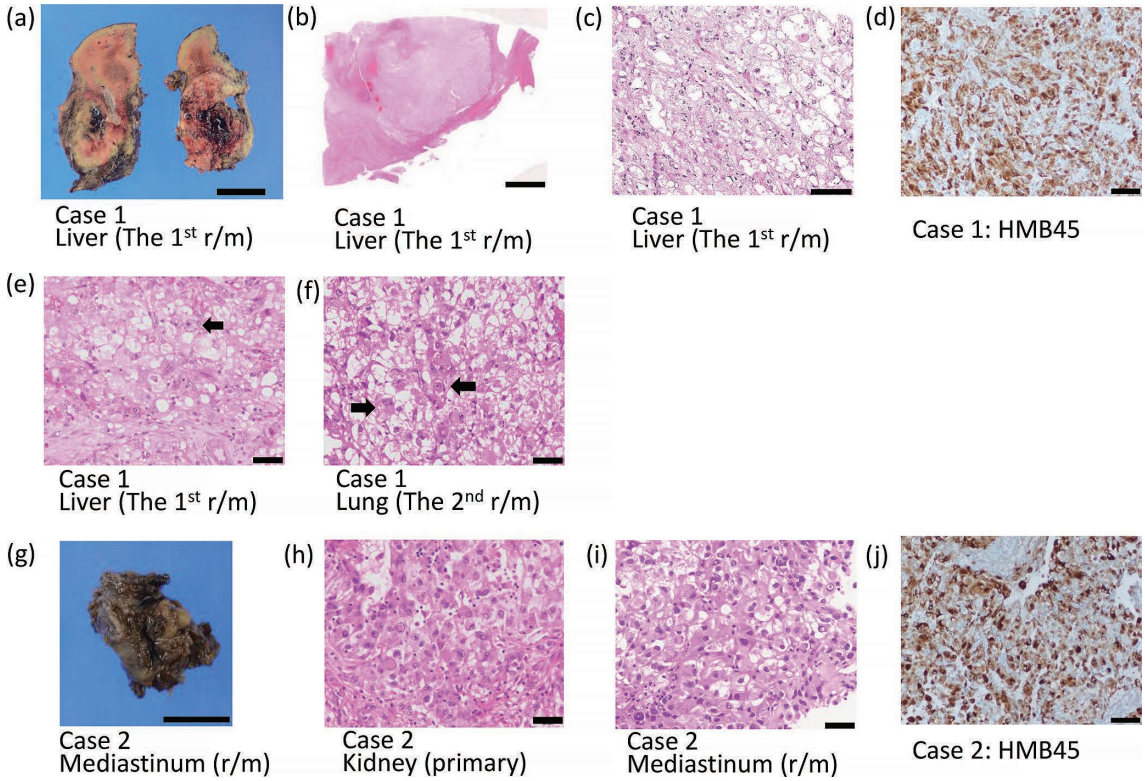

Fig. 2. Macroscopic and microscopic images of Case 1 and 2.
Case 1 (a-f): (a) Macroscopic appearance of the liver tumor, the 1st recurrence/metastasis (r/m); a yellowish, partially necrotic and hemorrhagic mass (bar = 3 cm). (b) Microscopically, the liver tumor demonstrated monotonous appearance [hematoxylin and eosin (HE) stain, loupe view, bar = 5 mm]. (c) The liver tumor consisted of a sheet of spindle cells and epithelioid cells (HE stain, middle-power-view, bar = 100 μm). (d) The tumor cells were positive for Human melanin black 45 (HMB45) (bar = 100 μm). (e)(f) Atypical epithelioid cells (arrow) were observed in the liver tumor (e), and the lung tumor, the 2nd r/m. (f) (bar = 50 μm).
Case 2 (g-j): (g) Macroscopically, the mediastinal tumor was measuring 2 cm (bar = 1 cm). (h)(i) Hematoxylin and eosin (HE) stain or tumors. (h) The renal tumor and (i) the mediastinal tumor, r/m, were composed of epithelioid cells. (j) The tumor cells were positive for HMB45.

Table 2. Summary of common immunohistochemical characteristics of EAML, clear cell renal cell carcinoma, and results of immunohistochemistry in Case 1 and Case 2.

	EAML	CCRCC	Case 1	Case 2
AE1/AE3	–	+	–	–
CD10	–	+	–	–
Melan A	+	–	+	+
HMB45	+	–	+	+

–, negative; +, positive; EAML, epithelial angiomyolipoma; CCRCC, clear cell renal cell carcinoma; HMB45, human melanin black 45.

and epithelioid cells without any foci of differentiation into adipose tissue and smooth muscle. The tumor cells were immunohistochemically positive for Melan A and human melanin black 45 (HMB45); common markers of PEComa family, including angiomyolipoma (Fig. 2d). We also confirm the tumor cells were negative for AE1/AE3 and CD10 to exclude epithelial tumors, in particular, clear cell renal cell carcinoma, which shows immunopositivity for both AE1/AE3 and CD10. These immunohistochemical findings are summarized in Table 2. There were several atypical epithelioid cells in the liver and lung lesions (Fig. 2e, f), but those cells did not reach 70% of the whole tumor. Mitotic figure counts were within 1/10 high power field (HPF) in all these tumors examined and there were no apparent atypical mitotic figures detected. The lesions in the kidney and liver had necrosis. The lesion in the lung demonstrated carcinoma-like growth patterns (Nese et al. 2011; Moch et al. 2016). External extension and involvement of renal vein

were not detected in all the lesions examined. The pathological features proposed by Brimo et al. (2010) and Nese et al. (2011) as predictive prognostic models were summarized in Table 3. However, both of the predictive models did indicate that all the lesions in this case were determined as low risk for progression despite aggressive clinical behavior.

Case 2

Macroscopically, all primary and recurrent/metastatic lesions demonstrated well-circumscribed nodules measuring up to 13 cm in the kidney and 2 cm in the mediastinum (Fig. 2g). Histologically, both of these two tumors were composed of epithelioid cells (Fig. 2h, i), immunohistochemically positive for Melan A and HMB45 (Fig. 2j) and negative for AE1/AE3 and CD10 without differentiation into adipose tissue and smooth muscle. There were many atypical epithelioid cells but their proportions did not reach 70% of the whole tumor. Mitotic figure counts were 4/10 HPF and 1/10 HPF in renal and mediastinal lesions,

Table 3. Summary of pathological and immunohistochemical findings of Case 1 and Case 2.

		Case 1		Case 2		
		Kidney	Liver (The 1st r/m)	Lung (The 2nd r/m)	Kidney	Mediastinum (r/m)
Pathological characteristics						
Nese's model	TSC and/or classical AML	–	–	–	+	–
	Tumor size (> 7cm)	+	–	–	+	–
	Extrarenal extension and/or involvement of renal vein	–	–	–	+	
	Carcinoma-like growth pattern	–	–	+	+	+
	Necrosis	+	+	–	+	–
Brimo's model	≥ 70% atypical epithelioid cells	–	–	–	–	–
	≥ 2 mitotic figures/10 HPF	–	–	–	+	–
	Atypical mitotic figures	–	–	–	+	–
	Necrosis	+	+	–	+	–
Results of immunohistochemistry						
	MDM2	–	+	++	–	++
	β-catenin (nucleus)	–	–	–	–	–
	GATA3	–/+	–	+	–	–
	p16	–/+	+	+	++	++
	cyclin D1	–/+	–	–/+	+	+
	CDK4	–/+	+	–/+	+	–
	c-kit	+	–/+	–/+	+	+
	EGFR	+	–	++	–	–/+
	GLUT1	++	+	++	+	–/+
	HER2	–	–	–	–	–
	ER	–	–	–	–	–
	PgR	–	–	+	–	+
	Bcl-2	–	–	–	–/+	+
	p53	–/+	–/+	–/+	–/+	–/+

–, negative; –/+, positive < 10%; +, 10-50%; ++, ≥ 50%; TSC, tuberous sclerosis complex; AML, angiomyolipoma; HPF, high-power field; MDM2, mouse double minute 2 homolog; GATA3, GATA binding protein 3; CDK4, cyclin-dependent kinase 4; EGFR, epidermal growth factor receptor; GLUT1, glucose transporter 1; HER2, human epidermal growth factor receptor type2; ER, estrogen receptor; PgR, progesterone receptor; Bcl-2, B-cell lymphoma 2; r/m, recurrence/metastasis.

respectively. There were atypical mitotic figures in the renal lesion, whereas no atypical mitotic figures in the mediastinum lesion. Both of these lesions in the kidney and mediastinum demonstrated carcinoma-like growth pattern. External extension and involvement of renal vein were also detected in the renal lesion. Case 2 met all five parameters of Nese's model of predicting aggressive clinical behavior of renal EAML and three of four parameters of Brimo's model, resulting in diagnosis as high risk for progression in both models.

Immunohistochemical analysis on cell cycle modulators and cell proliferation factors

We performed immunohistochemical analysis of the factors modulating cell cycle and compared the results between primary and recurrent/metastatic lesions. We targeted on 14 factors which have previously reported to be associated with malignancy and cell proliferation in other tumors as summarized in Table 3; β-catenin, GATA binding protein 3 (GATA3), p16, cyclin D1, Mouse double minute 2 homolog (MDM2), Cyclin-dependent kinase 4 (CDK4), c-kit, epidermal growth factor receptor (EGFR), Glucose transporter 1 (GLUT1), Human epidermal growth factor receptor type2 (HER2), Estrogen receptor (ER), Progesterone receptor (PgR), B-cell lymphoma 2 (Bcl-2), and p53. p53 mutations or c-kit underexpression was reported to be associated with recurrence or metastasis of AML (Nguyen et al. 2008; Li et al. 2012). ER and PgR are known to express in tumors of PEC family, and ER was reported to express more in EAML than in conventional AML (Cho et al. 2004).

In Case 1, MDM2 was immunohistochemically negative in the primary renal tumor (Fig. 3a), partially positive in the first metastatic liver tumor (Fig. 3b), and diffusely and intensely positive in the second metastatic lung tumor (Fig. 3c). Normal tissue adjacent to tumor in liver and lung was immuonegative for MDM2. The status of MDM2 immunoreactivity in the tumors of Case 2 was similar to that of Case 1, i.e., MDM2 negative in the primary renal tumor (Fig. 3d) and normal tissue adjacent to tumor in kidney, and diffusely positive in the metastatic mediastinal tumor (Fig. 3e). There were no significant differences between primary and metastatic lesions of EAML in other markers examined as above (Table 3). In every tumor of two cases, the expression pattern of p53 was spotty and weakly positive, known as p53 wild-type pattern.

The status of MDM2 immunoreactivity was significantly higher in metastatic than primary tumors as described above ($P = 0.0179$, Fisher's exact test) but there were no significant differences of Ki-67 labeling index (LI) between primary and metastatic tumors. The average of Ki-67 LI was 6.7% in metastatic tumor and 6.1% in primary tumor ($P = 0.87$, Student's t-test). There was no significant correlation between MDM2 status and Ki-67 LI in the whole tumor ($P = 0.87$, Student's t-test).

We then evaluated Ki-67 LI in three different spots corresponding to 1 mm^2 each of MDM2 positive and MDM2 negative areas in order to explore the possible

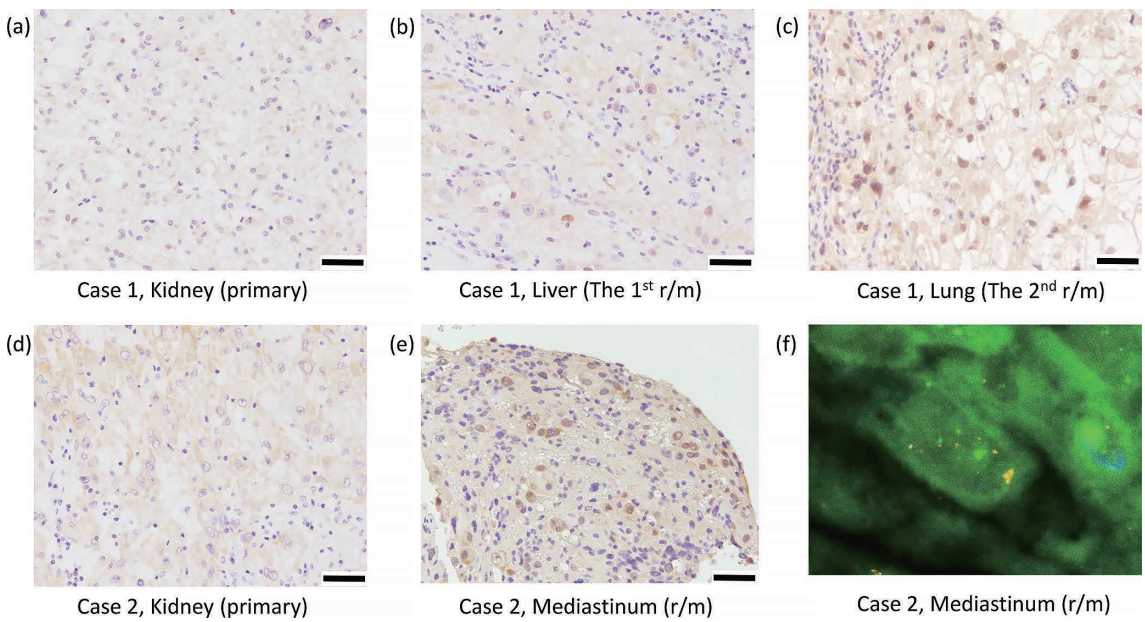

Fig. 3. Immunohistochemical study of mouse double minute 2 homolog (MDM2).
Immunohistochemical study of MDM2 (bar = 50 μm) and FISH for MDM2.
Case 1 (a-c): (a) MDM2 was negative in the primary renal tumor, (b) partially positive in the liver tumor, the 1st recurrence/metastasis (r/m), and (c) diffusely positive in the lung tumor, the 2nd r/m. Case 2 (d,e): (d) MDM2 was negative in the renal tumor, and (e) diffusely positive in the mediastinal tumor, the 1st r/m. FISH for MDM2 in mediastinal tumor (the 1st r/m) of case 2 (f): MDM2 amplification was observed. MDM2: spectrum orange, CEP12: spectrum green.

correlation between MDM2 abnormalities and increased cell proliferation. We compared the mean value of Ki-67 LI in MDM2 positive and negative areas of all the lesions in each case using Student's t-test. In case 1, Ki-67 LI in MDM2 positive area was significantly higher than MDM2 negative area (P = 0.0001) (Table 4). However, in case 2, no significant differences of Ki-67 LI were detected between MDM2 positive and negative areas (P = 0.23). In addition, we examined 3 cases without metastasis/recurrence (Case 3, 4, 5) of EAML surgically resected at Tohoku University hospital in 2010-2015, harboring no recurrence or metastasis for at least one year. We also immunolocalized MDM2 and Ki-67 in these 3 cases (Table 5). All three cases were immunohistochemically negative for MDM2.

MDM2 FISH

We performed FISH on metastatic/recurrent three tumors of Case 1 and Case 2, which were immunopositive for MDM2. MDM2 amplification was observed in metastatic mediastinal tumor of Case 2 (Fig. 3f). In liver and lung tumors of Case 1, we observed some tumor cells with MDM2 amplification; however, MDM2/CEP12 ratio was lower than the threshold.

Discussion

In this study, we firstly evaluated the status of proteins associated with cell proliferation in EAML and compared the results between primary and metastatic/recurrent tumors in two cases of EAML. We did demonstrate possible involvement of MDM2, which was associated with metastasis/recurrence of EAML in these semi-comprehensive analyses, in clinical progression of EAML. Putative relationship between progression of EAML and MDM2 expression are illustrated (Fig. 4).

There are two previous reports showed that MDM2 immunoreactivity in AML tissue. Asch-Kendrick et al. (2016) showed that MDM2 positivity was observed in three fat-predominant AML (3/13 cases) one conventional AML (1/14 cases) and one epithelioid AML (1/3 cases). Lin et al. (2018) showed that focal expression of MDM2 was observed in 10 (40%) AML cases, and diffuse and focal

Table 4. Ki-67 labeling index (LI) in positive and negative areas.

	MDM2	Ki-67 LI (%)	Organ	Ki-67 LI (%)	Comparison of Ki-67 LI: Positive area vs negative area for MDM2
Case 1	positive	10.4 ± 2.5	liver	10.7 ± 3.5	
			lung	10.1 ± 1.8	
	negative	2.4 ± 2.0	kidney	2.3 ± 1.7	
			liver	2.6 ± 2.6	P = 0.0001
Case 2	positive	6.2 ± 5.3	mediastinum	6.2 ± 5.3	
	negative	10.7 ± 4.7	mediastinum	10.8 ± 3.9	
			kidney	10.6 ± 6.3	P = 0.23

In each case, the mean of Ki-67 LI in MDM2 positive and negative areas of all lesions were compared using Student's t-test. Data were presented as mean ± standard deviation. MDM2, mouse double minute 2 homolog; Ki-67 LI, Ki-67 labeling index.

Table 5. MDM2 expression and Ki-67 labeling index (LI) in each lesion.

	Organ		MDM2	Ki-67 LI (%)
Case 1	kidney	primary	–	2.3
	liver	The 1st r/m	+	7.2
	lung	The 2nd r/m	++	10.1
Case 2	kidney	primary	–	10.6
	mediastinum	r/m	++	8.4
Case 3	liver	primary	–	6.7
Case 4	kidney	primary	–	0.71
Case 5	kidney	primary	–	12.2

–, negative; –/+, positive < 10%; +, 10-50%; ++, ≥ 50%; MDM2, mouse double minute 2 homolog; Ki-67 LI, Ki-67 labeling index; r/m, recurrence/metastasis.

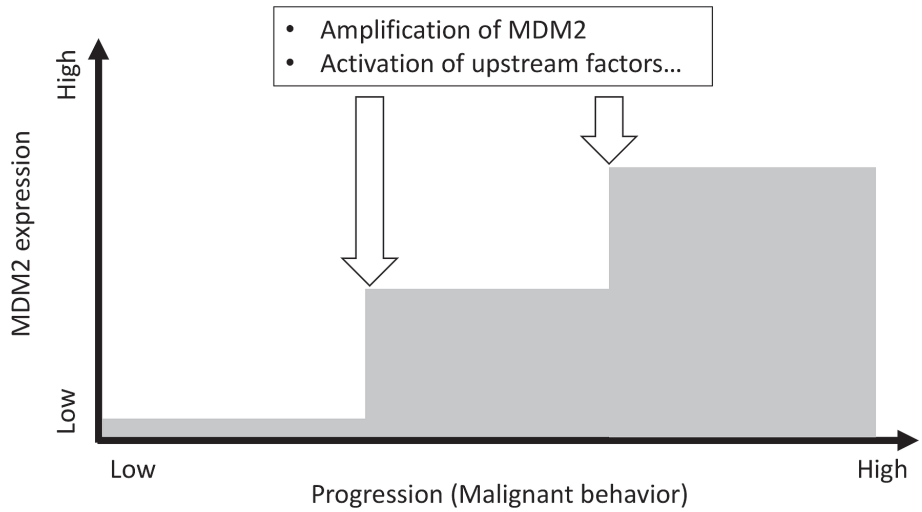

Fig. 4. Summary of putative relationship between MDM2 expression and progression behavior of EAML. MDM2 overexpression through amplification, activation of upstream factors, and others could possibly influence the progression of EAML.

expression of p16 was observed in 15 (60%) AML cases. In both of the two reports, the authors focused on distinguishing liposarcoma from AML, and did not perform any analyses about if MDM2 or p16 work for AML progression or not. Furthermore, conventional AML is known to a common, benign tumor, although EAML is known to malignant potential tumor, and one-third of them have been reported to show recurrence and/or metastases. EAML is listed separately from AML in World Health Organization (WHO) classification of renal tumors (Moch et al. 2016). EAML is assumed to have different biological characterization from conventional AML (Mete and van der Kwast 2011; Varma et al. 2011). Therefore, we should consider the possibility that MDM2 and p16 in EAML may play different roles from conventional AML. In this report, MDM2 protein expression increased stepwise through every metastasis/recurrence in Case 1, and also increased in metastatic tumor of Case 2. Overexpression of MDM2 has been reported in many malignant tumors (Momand et al. 1998). In addition, MDM2 expression has been reported to be correlated with progression of tumors, such as nonfunctioning pituitary adenoma (Yao et al. 2017). Increased MDM2 expression was more frequently detected in seminoma and embryonal carcinoma than intratubular germ cell neoplasia (Datta et al. 2001). MDM2 may play important roles in molecular mechanisms of EAML progression as same as these other tumors. In this study, we also performed MDM2 FISH, and we demonstrated significant amplification of MDM2 gene in metastatic/recurrent tumor of Case 2. We also observed MDM2 gene amplification in some tumor cells in metastatic/recurrent tumors of Case 1. Therefore, we considered that MDM2 gene amplification may have enhanced MDM2 expression in these cases, especially in Case 2. However, there may be the possibility that other mechanisms, such as overexpression or activation of upstream factors of MDM2, could take part in upregulation of MDM2, so genetic examinations in more detail should be performed in the future.

MDM2 is well known to promote cell-cycle suppressor p53 in normal cells. The immunoreactivity of p53 showed wild-type pattern in every tumors. Immunoreactivity of p53 do not always reflect volume of p53 protein in tumor cells, hence we could not evaluate correctly correlations between MDM2 and p53 expression in the EAML tissue. Recently, p53-independent effects of MDM2 such as regulation of many cellular processes including cell-cycle, apoptosis, genome instability, epithelial-mesenchymal transition, and others attract attentions have been also reported (Bohlman and Manfredi 2014; Fåhraeus and Olivares-Illana 2014; Urso et al. 2016). In Case 1, Ki-67 LI gradually increased in metastatic/recurrent tumor as in MDM2, which is consistent with the hypothesis that MDM2 could promote the cell proliferation of tumor cells in EAML. However, in Case 2, Ki-67 LI decreased in metastatic tumor despite increased MDM2 status. In addition, there were no statistically significant correlations between MDM2 status and Ki-67 LI in each lesion. There have been no reports regarding the direct comparison between MDM2 status and Ki-67 LI but MDM2 expression is reasonably postulated not to be directly correlated with cell proliferation of the tumor cells because factors involved in the process other than MDM2 could modulate the mechanisms of cell proliferation (de Souza et al. 1999; Hashimoto et al. 2000; Khor et al. 2009). In addition, MDM2 could influence not only cell proliferation but also other cellular processes following to tumor development. In this report, we had only two EAML cases with metastasis/recurrence because EAML itself is rare. Further investigations involving a greater number of EAML cases is needed in order to clarify the clinical and biological significance of MDM2 in EAML.

In summary, this study has demonstrated that MDM2 could possibly influence the progression of EAML and

could provide new insights into the novel prognostic factors and/or therapeutic targets of the patients with EAML.

Acknowledgments

The authors thank Mr. Katsuhiko Ono (Tohoku University School of Medicine) and Ms. Yayoi Aoyama (Tohoku University Hospital) for their excellent technical support, and thank Dr. Yasuhiro Miki for advice on the manuscript.

Author Contributions

C.I. and R.S. evaluated pathological findings and immunohistochemistry, performed the pathological analyses, and wrote the initial draft of the manuscript. W.N., H.K., and S.E. collected tissue samples and supervised the interpretation of clinical data. F.F. and M.W. checked the data and pathological diagnosis, and edited the manuscript. H.S. supervised all data of the pathological diagnosis and the analyses, and edited the manuscript. All authors approved the submission of the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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