学位論文

Association of D2-40 and MMP-1 expression with cyst formation in lung metastatic lesions of cutaneous angiosarcoma on the scalp: immunohistochemical analysis of 23 autopsy cases

(頭部血管肉腫の嚢胞型肺転移における D2-40 と MMP-1 発現：
剖検 23 例の免疫組織学的検討)

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著者の宣言

本学位論文は著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

要旨

背景：血管肉腫は高齢者の頭部皮膚に好発する、非常に稀な血管内皮細胞由来の悪性腫瘍である。頭部の皮膚血管肉腫は早期に肺転移をきたし、多くは血胸、気胸を併発するため致死的となる。加えて、血管肉腫の肺転移巣は、他の悪性腫瘍の肺転移ではみられることの少ない、“薄壁空洞”とよばれる嚢胞構造を形成することが特徴的である。肺表面に薄壁空洞が出現した場合、これが破裂することによって再発性、難治性の気胸を生じ、治療不可能な呼吸不全を招く結果となる。

目的：この薄壁空洞の発生機序について検討することが、今後の血管肉腫の治療戦略を立てる上で重要と考え、頭部血管肉腫患者で肺転移を認めた 23 例の剖検例について解析を行った。

方法：1985 年から 2011 年の間に北里大学病院皮膚科で治療され、死亡後に病理解剖を行った頭部血管肉腫のうち、肺転移を認めた 23 例の画像所見および病理組織学的所見について検討した。初診時に採取された原発病変および剖検時に得られた肺、肝転移病変に対して、リンパ管内皮細胞マーカーである D2-40、血管内皮細胞マーカーである ERG 抗体、タンパク分解酵素として MMP-1, MMP-2, MMP-7, MMP-9 について免疫組織学的検討を行った。

結果：23 例の年齢は 59-94 歳（中央値 74 歳）で、男女比は 16：7 であった。肺転移巣の CT 画像所見による分類では、薄壁空洞型と結節型がそれぞれ 9 例（39%）、薄壁空洞と結節の混合型が 3 例（13%）、すりガラス状陰影型が 2 例（9%）であり、78％で薄壁空洞型または結節型のどちらかの所見を呈した。また薄壁空洞型では 1 例を除くすべての症例で気胸を併発した。病理組織学的検討においては、HE 染色では、薄壁空洞病変で空洞内腔を取り囲む腫瘍細胞は認識困難であったが、D2-40 染色により腫瘍細胞の分布を明らかに確認することが可能であった。この D2-40 染色は、皮膚原発巣および肺転移巣のいずれにおいてもそれぞれ 100%、92%と高率に陽性であった。ERG 発現も D2-40 と同様に原発巣および肺転移巣に高率に陽性であったが、同時に正常血管内皮細胞にも陽性となるため、腫瘍細胞の分布の検出には D2-40 がより有用であった。MMP-1 発現は原発巣および肺転移巣においてそれぞれ 95%、82.6%と陽性であった。MMP-1 の発現は、薄壁空洞形成に関与すると思われたが、空洞型と結節型で MMP-1 発現の程度に差はなかった。MMP-2 および MMP-7 の発現はほぼ見られず、MMP-9 は肺転移の 29%に陽性であった。

考察：血管肉腫の薄壁空洞型の肺転移の発生機序については、従来、下記の４つの仮説が考えられている。１）既存の空洞への腫瘍細胞浸潤、２）細気管支に併走する細血管からの腫瘍細胞浸潤により、細気管支が不完全に閉塞し、チェックバルブ機構によって末梢の肺胞が崩壊し空洞形成する、３）腫瘍細胞塊

の中心部に生じた壊死が、貪食細胞による消化や経気管支的に排出され空洞化する、４）腫瘍細胞が分泌するタンパク分解酵素により肺胞が破壊され空洞化する。我々の経験した 23 例と過去の報告すべてで、肺転移前に既存の空洞が認められた症例はなかった。チェックバルブ機構については、複数の症例報告で仮説を支持した報告がある。彼らの考察では、病理学的に空洞の周囲に腫瘍細胞が認められないことがチェックバルブ説を支持する理由となっているが、それを裏付ける根拠はない。今回検討した薄壁空洞型の 13 例すべてにおいて、空洞の周囲を取り囲むように腫瘍細胞を認めている。また、肝転移例でも肺転移と同様の多発する嚢胞性転移巣がみられたことは、チェックバルブ説では説明できない。中心壊死からの空洞形成説については、我々の経験した急速進行型の 1 例で、CT 画像上、小結節として出現した肺転移が、1 ヶ月後に空洞化した。さらに病理組織学的にも、腫瘍塊の中心に組織球で囲まれる壊死像を認めた。このような病理所見は他の薄壁空洞型では見られなかったが、一部の急速進行型に限っては、中心壊死が薄壁空洞形成の原因となりうると考えられた。最後に、タンパク分解酵素の関与については未だ推測の域をでないものの、この研究で、我々は頭部血管肉腫の原発巣および肺転移巣に高率に MMP-1 が陽性になることが示された。また、多くの症例で CT 画像にて薄壁空洞が前病変なしに突如出現し、初期にはハローを伴わないこと、病理組織学的に空洞は 2-3 層の腫瘍細胞で取り囲まれており、壊死性変化はほとんどみられないことから、MMP-1 のような蛋白分解酵素が薄壁空洞形成に関与している可能性が示唆される。今後、空洞形成の主因を担う蛋白分解酵素の同定には、さらなる研究が必要である。

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1. Introduction

Cutaneous angiosarcoma is a very rare but is an aggressive malignant vascular tumor. It commonly occurs on the scalp and face in the elderly. The overall survival rate of 5 years is approximately 20% [1]. The lung is the most frequent metastatic site of cutaneous angiosarcoma. Lung metastasis causes the majority of deaths due to respiratory complications. Although the primary site in most cases is treated with multimodality therapy consisting of irradiation, chemotherapy, and/or surgical removal, lung metastasis is still uncontrollable with current therapies [2,3]. Therefore, it is important to clarify the intrinsic mechanism of lung metastasis to develop a treatment strategy. The appearance of lung metastasis, in these cases, can be classified into 2 types: cystic and nodular. In particular, the surface, cystic type tends to cause pneumothorax by rupturing [4–6]. Although it has a unique appearance as a thin-walled cyst in the radiological examination [7], the tumor cell behavior causing the cystic lesion remains unknown. The check-valve mechanism, hollowing from a central necrosis and participation of an auto-degeneration enzyme as the hypothesis, is still under discussion. Here, we analyzed 23 autopsy cases of angiosarcoma on the scalp with lung metastasis to reveal the mechanism of developing cystic pulmonary metastasis. We focused on proving the existence of tumor cells in cystic metastatic lesions mainly by D2-40 immunostaining and examining the role of matrix metallo-proteinase (MMP) as an auto-destruction enzyme in developing a thin-walled cyst.

2. Materials and methods

2.1. Patients and tissue samples

Twenty-three cases of angiosarcoma of the scalp with metastases to the lung parenchyma and/or pleura metastases were collected from the autopsy file of Kitasato University Hospital from 1985 to 2011. The clinicopathological findings of these 23 cases are summarized in Table 1. Basically, combined modality therapy was provided, although some cases had a single therapy because of their disease stages or underlying diseases. Immunotherapy with recombinant interleukin-2 was performed by local injection, systemic administration, or arterial administration. Low-dose docetaxel or paclitaxel was administrated as chemotherapy, either weekly or biweekly. The total radiation dose was 70 to 80 Gy of electron beams for primary lesions on the scalp, and 60 Gy of x-ray beams for metastatic lymph nodes. The fractions were 2 to 2.5 Gy daily. Surgery was performed as a minimal extent resection to reduce the tumor mass. Tissue

samples of scalp biopsies from primary lesions and pulmonary metastatic lesions were collected during autopsies from all 23 cases. Twenty-one cases of primary lesions were analyzed, but the 2 cases in which the primary lesions had been resected in the previous medical institutions were not. Twenty-five samples of lung metastatic lesions were analyzed from 23 cases; only cases 1 and 3 provided two samples, because they had both nodular and cystic lesions. All radiological lung findings were assessed by chest computed tomography (CT) when the patients were alive, and all tissue samples of pulmonary lesions were obtained from the autopsies. In 5 cases, tissue samples of liver metastasis were also collected from autopsy samples. In addition, tissue samples of capillary hemangioma and cavernous hemangioma from 10 cases each were used for purposes of comparison as benign hemangioma. Each sample was routinely fixed with 10% formalin and embedded in paraffin, and histologic sections were stained with hematoxylin-eosin (HE) for immunohistochemistry.

2.2. Immunohistochemical staining and analysis

Immunohistochemical staining of 4-μm-thick paraffin sections was performed using an Envision^{+} kit (DakoCytomation, Glostrup, Denmark). The primary monoclonal antibodies and their working dilutions are shown in Table 2. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 15 minutes. The sections were then incubated with the primary antibodies at the given dilutions in PBS (phosphate-buffered saline) overnight at 4°C. 3,3'-diaminobenzidine was applied as the final chromogen, and nuclei were counterstained with hematoxylin or methylgreen solution to facilitate histopathological assessment. Appropriate positive controls were stained in parallel. MMP-1, 2, and 9 staining of parietal cells were used as positive controls [8]. Previously confirmed positive control slides of a colon cancer lesion were used for the positive control for MMP-7 [9]. Staining of lymphatic endothelium was used for an internal positive control of D2-40 expression. Vascular endothelium was used as an internal positive control for the estrogen-regulated gene (ERG). The expression profiles were analyzed according to a 4-tiered system (negative, no positive cells; equivocally stained, most cells are very slightly positive; focal positive, a few parts of the tumor are strongly stained; diffuse, the whole tumor is strongly stained). CD68-stained slides of lung metastasis were used to examine the distribution of macrophages.

2.3. Western blot analysis for the specificity of MMP-1

The human hemangiosarcoma cell line (ISO-HAS) [10] and the normal human dermal

fibroblast cell line (KF-4009) (Kurabo, Osaka, Japan) were maintained as monolayers in DMEM (Dulbecco's modified eagle's medium) supplemented with 10% heat-inactivated FBS (fetal bovine serum) and 50 μg/mL gentamicin (Gibco, Invitrogen, Carlsbad, CA). For Western blot analysis, total proteins from subconfluent monolayers of these cells were collected in lysis buffer (20 mmol/L Tris–HCl [pH 7.5], 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 10% glycerol, 100 mmol/L phenylmethylsulfonyl fluoride, protease inhibitor cocktail). The supernatants were recovered for determination of proteincontent. Aliquots of proteins (50 μg/well) were subjected to 10% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and then transferred to Immobilon-P (Millipore Corporation, Bedford, MA), which were blocked in 1% (w/v) BSA (bovine serum albumin). A specific protein on the membranes was detected by incubation with an anti-MMP-1 primary antibody (diluted 1:200, Daiichi Fine Chemical, Takaoka, Toyama, Japan), followed by peroxidase-conjugated Protein A/G (Thermo Scientific Peirce Recombinant Protein, Rockford, IL) with ECL plus a Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK).

2.4. Statistical analysis

Differences between the groups were evaluated by the Fisher exact test for categorical variables. Analyses were performed using the SPSS version 12.0 statistical software (SPSS Inc, Chicago, IL). P b .05 was considered as statistically significant. This study, using clinical information and pathological samples from the Kitasato University Hospital, was approved by the Kitasato University School of Medicine and Kitasato University Hospital Ethics Committee (B12-136).

3. Results

3.1. Patients' summary

The age of the patients ranged from 59 to 94 years (median age, 74 years) (Table 1). The male-to-female ratio was 16:7. Lung metastasis detected in the last chest CT scan prior to the patient's death was classified in four types: only the thin-walled cystic type, 9 cases (39%); only the nodular type, 9 cases (39%); the cystic and nodular mixed type, 3 cases (13%); and the GGO (ground-glass opacity) type, 2 cases (9%). The findings of pulmonary metastasis using a CT scan were divided into 2 main types, cystic and nodular. Basically, the findings were not mixed cyst and nodule, only 3 cases (cases 1, 3, and 11) had both findings. In almost all the cases with cystic lesions, there were not any pre-existing lesions before the cysts developed, although a CT scan was performed

every 3 months (Fig. 1A and B). However, in case 1, a few tiny nodular lesions that had been identified by CT scan changed to thin-walled cysts with halos in 6 weeks (Fig. 1E and F). Eighteen cases (78%) presented with pleural invasion in the lung tissue. With one exception, all the cases had cystic metastasis-complicated pneumothorax, which became a leading cause of the patients' deaths. Liver metastasis was revealed in 8 cases; and of those, 5 cases were histologically analyzed. The CT images of the metastatic lesions were observed as nodules or cystic lesions (Fig. 2A).

3.2. Immunohistochemical staining

In primary and pulmonary metastatic lesions of scalp angiosarcomas, D2-40 immunoreaction was 100% and 92% positive, respectively (Tables 3 and 4). All primary lesions and all but 2 lung metastatic lesions were diffuse or focal positive. The most common cystic metastatic lesions of the lung were clear cavities without necrotic changes or fibroses, and it was difficult to detect tumor cells around the cyst under low magnification (Fig. 1C). However, the tumor cells lining the cyst were clearly observed by D2-40 staining (Fig. 1D, arrowheads). As a rare case, necrotic change was observed in the cystic metastasis in Case 1, and there were denser tumor cells, positive for D2-40, in the cyst wall (Fig. 1G and H). All liver metastatic lesions but one were also diffuse or focal positive for D2-40. In the cystic type of liver metastasis, tumor cells were observed on the inside wall by D2-40 staining as well as cystic metastasis of the lung (Fig. 2B and C). However, D2-40 immunoreactions were all negative in capillary hemangiomas and cavernous hemangiomas. Distinct nuclear staining for the ERG expression was observed in 20 of 21 primary lesions (95%), in 23 (92%) of 25 pulmonary metastatic lesions, in 1 (20%) of 5 liver metastatic lesions, and in all benign hemangiomas. MMP-1 expression was also frequent and uniform in primary lesions (95%) and pulmonary metastatic lesions (83%). In hepatic lesions, 3 (60%) of the 5 cases were positive. There were no significant differences of MMP-1 expression between the cystic and nodular types of pulmonary metastasis. Fig. 3 shows tumor cells in the primary lesion and the lung. The pleomorphic and spindle-shaped tumor cells in the HE stain were also positively stained for D2-40, ERG, and MMP-1. An MMP-9 expression was observed in a small number of cases: 6 (29%) of 21 cases of primary lesions, and 4 cases of pulmonary lesions in 25 samples (16%) from 23 cases. All hepatic lesions were negative for MMP-9. Moreover, MMP-2 and MMP-7 were negative in either primary or metastatic lesions for almost all the cases. Regarding benign hemangiomas, MMP-1 was positive in 5 cases (50%) of capillary hemangiomas and 1 case (10%) of cavernous hemangioma (significantly low, P b .01, P b .001,

compared with primary lesions of angiosarcoma, respectively). MMP-9 was positive in only 1 case (10%) of cavernous hemangioma. MMP-2 and MMP-7 were all negative in both hemangiomas. Infiltration of macrophages localized around the pulmonary metastatic lesion was seen in 5of the cystic cases (42%) and in 4 of the nodular cases (31%). In more than half of the cases, macrophages infiltrated diffusely throughout the lung, not only around the metastatic lesions, in association with intra-alveolar hemorrhage (data not shown).

3.3. Western blot analysis of cultured cells

To determine the MMP-1 expression in angiosarcoma cells, the expressions MMP-1 and β-actin were examined after incubation for 24 hours by Western blot analysis using the ISO-HAS cell line. The KF-4009 cell line was run as a positive control of MMP-1. Anti-mouse β-actin was used as a loading control. MMP-1 was expressed in the ISO-HAS and KF-4009 cell lines (Fig. 4).

4. Discussion

Pneumothorax is a common symptom of pulmonary metastasis in patients with cutaneous angiosarcoma and is usually uncontrollable in relapses and bilateral cases [6,7,11]. In the present study, 11 of 12 cases that showed cystic lesions of the lung resulted in pneumothorax and led to lethal respiratory failure. The pneumothorax must occur for growing and rupturing of thin-walled cysts shown by the CT scans. However, the pathogenesis of thin-walled cysts remains unknown. We investigated how tumor cells can be identified in the cystic walls and how the pulmonary metastasis generates such thin-walled cysts. For the first question, regarding how tumor cells can be identified in the cystic walls, D2-40 immunopositive cells were clearly identified in the cyst wall as angiosarcoma cells by immunohistochemical analysis (Fig. 1D and H). The monoclonal antibody against D2-40, Mr 40,000 O-linked sialoglycoprotein, used in the present study, reacts with a fixation-resistant epitope in the lymphatic endothelium. D2-40 is well known as a lymphatic marker and is reported to be a possible marker for angiosarcoma in 31% to 53% of cases [12–15]. Moreover, limited to the reports for only cutaneous angiosarcoma, D2-40 was positive at a much higher rate (90%-100%) [16,17]. CD31 and CD34 are also well known as endothelial markers and could be positive for angiosarcoma cells. However, CD34 tends to be negative for angiosarcoma cells compared with D2-40 [18].Recent studies have shown that ERG, an ETS (erythroblast transformation-specific) family transcription factor, is a sensitive marker of angiosarcoma and other vascular tumors [19]. In the present study, ERG was positive in

all but 1 primary lesion of angiosarcomas and all benign hemangiomas. However, in the pulmonary and hepatic metastatic lesions, the ERG positive rate was relatively lower (92% and 20%, respectively). One possibility for the lower figures might be that the metastatic lesions were obtained from autopsy samples, which may have a lower quality for immunohistochemistry, particularly livers. From our experience, because of the extensive endothelial labeling of the conventional endothelial markers, CD31 and CD34, it is difficult to differentiate angiosarcomas from benign hemangiomas and the background intensity of normal vessels. While there is a study in which D2-40 expression was noted in the alveolar lining in the normal adult lung [20], the expression was diminished or lost in the lung around the pulmonary metastatic lesions. In point of fact, D2-40 immunohistochemistry clearly demonstrated the metastatic tumor cells in lungs. Because all primary lesions and most pulmonary metastatic lesions were positive (100% and 92%, respectively) for D2-40 in the present study, D2-40 is a useful marker to detect tumor cells in both primary and metastatic lesions in cutaneous angiosarcomas on the scalp. For the second question, regarding how the pulmonary metastasis causes such thin-walled cysts, previous studies have enumerated four hypotheses [3,6,21–23]. The hypotheses of the putative mechanisms of cyst formation are: (1)invasion of the metastatic tumor into the pre-existing lung cyst wall, (2) peribronchiolar invasion of the tumor induces peripheral cystic formation by the check-valve mechanism, (3) central necrosis of the tumor mass because of ischemic change, and (4) production of an auto-destruction enzyme by the lung metastatic tumor. To begin with, the pre-existing cyst wall, implied in the first hypothesis, was not observed radiologically in any of the cases in the present study, and to our knowledge, no investigators support this hypothesis. Regarding the second hypothesis, the check-valve has been considered as a possible mechanism, e.g., a peripheral tumor produced partial bronchial obstruction and acted as a check-valve phenomenon causing pulmonary wall rupture. However, this is a theoretic and yet unproved mechanism. Sakurai et al [6] advocates the check-valve mechanism. Their reason was that thin-walled cysts lacked a tumor cell lining. In the present study, the most frequent pattern observed was a few layers of tumor cells lining the inner surface of the cyst as in Case 2 (Fig. 1D). The variation could be explained by a difference in the tumor invasive stage because some older cysts tended to be associated with a high infiltration of tumor cells. Moreover, we saw, in the present study, that hepatic metastasis of angiosarcoma tended to form a similar cystic lesion (Fig. 3). Therefore, it would be erroneous to apply the check-valve hypothesis to the cyst formation from pulmonary and hepatic metastases. In the present study, novel histological findings were observed in Cases 1 and 3, in which the tumor invasion was

very aggressive. Several metastatic nodules with central necrosis showed cystic changes histologically (Fig. 1G, H). Especially, in case 1, it was observed on a CT scan that a tiny nodule changed to a cystic lesion (Fig. 1E, F). It was likely that the rapid proliferation of the tumor caused the central necroses as these 2 patients died because of aggressive pulmonary metastasis that had spread rapidly for a few months. Although CD68-positive macrophages were observed histologically around the cysts in 42% of these cases, the macrophages existed sparsely in all the cases except Cases 1 and 3. Therefore, cavitation from necrosis with macrophages seems to be a minor pathway. Therefore, in a small number of cases, the central necrosis in the third hypothesis might explain the cyst formation. Regarding the fourth, hypothesis of auto-destruction, to our knowledge, the potential to produce some autodestruction enzyme from metastasized angiosarcoma cells has not yet been reported. We focused on lymphangioleiomyomatosis (LAM) is well known for showing multiple cystic lung destructiveness similar to the pulmonary metastasis of cutaneous angiosarcoma. Most patients with LAM present with pneumothorax caused by rupturing cysts on the lung surface [24]. Immunohistochemical studies suggested that excess MMPs synthesized by LAM cells functioned in the proteolytic mechanisms of this disease[25,26]. MMPs comprise a family of at least 25 extracellular matrix-degrading, zinc-dependent enzymes. MMP-2 and MMP-9 can degrade native type IV collagen, denatured type I collagen, and elastic fibers [27]. Previous immunohistochemical studies found that MMP-2 and MMP-9 are predominantly expressed in LAM cells, suggesting that they are involved in the destructive cystic formation in LAM. MMP-1 in LAM lung nodule is capable of degrading collagen and elastin that are principal components of the alveolar wall scaffolding in the lung parenchyma [24]. Furthermore, Takeuchi et al [28] reported a case of splenic angiosarcoma which showed MMP-1 expression in primary splenic lesions. In the present study, MMP-1 expression was found in almost all primary and metastatic lesions. MMP-1 was expressed consistently in angiosarcoma, and the expression did not change regardless of the lesion site or type of pulmonary metastasis. MMP-1 seems not to be related with cyst formation because there was no difference between cystic and nodular metastasis. For the immunostaining results of the benign hemangiomas, immature proliferative endothelial cells might tend to express MMP-1, judging from the results that 50% of capillary hemangiomas were positive, but only one case of cavernous hemangioma was positive.

5. Conclusions

In conclusion, D2-40 immunopositivity is a reliable marker to identify metastatic

angiosarcoma cells in the cystic walls of pulmonary metastatic lesions.
We propose the following mechanisms of cyst formation. As a major cause, it is conceivable that proteinases like MMP-1 are associated with angiosarcoma cell proliferation. Central necrosis is another possible cause of cyst formation in a small number of cases, particularly in rapidly progressive cases. Both high proteinase activity and tumor cell necrosis may be associated with cyst formation, while the contribution of necrosis may vary depending on the case.

6. Future experiment

Further studies are warranted to discover other proteinases that will explain the difference between cystic and nodular metastasis formation.

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9. Accomplishment lists

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10. Tables and figures

Table 1 Clinical summary of the cases

Case number	Age	Sex	Chest CT findings	Histlogical findings in lung			Lung complication		Other metastases	Therapy
				Cyst	Nodule	Pleura invasion	Pneumothorax	Pleural effusion		
1	94	F	Multiple TC+N	+*	+	+	+	+	Neck LNs	RT
2	73	M	Multiple TC	+	−	+	+	+	Liver, spleen	RT, CT
3	86	F	A few TC+N	+*	+	+	+	+	Skull, dura mater	RT
4	70	M	A few TC	+	−	+	+	+	Small intestine colon	S
5	76	M	A few TC	+	−	+	+	+	Bone, colon, spleen, pancreas, neck LNs	RT, CT
6	67	M	Multiple TC	+	−	+	+	+	Infraclavicular LNs	RT, CT
7	69	F	Multiple TC	+	−	+	+	+	Bone	S, IT
8	72	M	Multiple TC	+	−	−	+	+	None	RT, CT
9	80	M	Multiple TC	+	−	+	+	+	Liver, thyroid, neck LNs	IT, CT
10	83	F	TC	+	−	+	−	+	None	S, IT
11	72	M	Multiple TC	+	+	+	+	+	Gingiva, neck LNs	RT, CT
12	58	M	Multiple TC	+	−	+	+	+	Liver	S, RT, CT
13	59	F	N	−	+	+	−	+	Eye-ball, uterus, liver, bone, para-aortic LNs	IT, RT, CT
14	84	M	Small N	−	+	−	−	+	Liver, bone, neck LNs	S, IT
15	80	F	Multiple N	−	+	−	−	Unknown	Neck LNs	IT, RT
16	77	M	Multiple N	−	+	−	−	+	Liver, spleen, bone, adrenal gland, mesenteric LNs	S, IT
17	74	F	Multiple N	−	+	−	Unknown	+	None	IT
18	74	M	Small N	−	+	+	−	+	None	IT
19	62	M	Multiple N	−	−	+	−	+	Liver	RT, CT
20	68	M	GGO	−	−	+	−	+	Bone, neck LNs	IT, RT, CT
21	79	M	N	−	+	+	−	+	None	S, IT
22	65	M	GGO	−	+	+	−	+	Liver, bone, neck LNs	S, IT, RT, CT
23	80	M	Small N	−	−	+	−	+	Kidney	S

Abbreviations: M, male; F, female; TC, thin-walled cyst; N, nodule; GGO, ground-glass opacity; LNs, lymph nodes; RT, radiation therapy; CT, chemotherapy; IT, immune therapy; S, surgery

NOTE. * cyst with necrotic change.

Table 2 Primary antibodies used in the present study

Antibodies	Clone	Dilution	Source
D2-40	D2-40	1:50	Dako Cytomation, Glostrup, Denmark
ERG	ERP3864	1:200	Novus Biologicals, Littleton, Colorado, USA
MMP-1	41-1E5	1:100	Daiichi Fine Chemical, Takaoka, Toyama, Japan
MMP-2	42-5D11	1:100	Daiichi Fine Chemical, Takaoka, Toyama, Japan
MMP-7	141-7B2	1:200	Daiichi Fine Chemical, Takaoka, Toyama, Japan
MMP-9	56-2A4	1:50	Daiichi Fine Chemical, Takaoka, Toyama, Japan
CD68	PG-M1	1:100	Dako Cytomation, Glostrup, Denmark

Table 3 Immunohistochemical findings of the primary and metastatic lesions

Case no.	Lesion type	D2-40	ERG	MMP-1	MMP-2	MMP-7	MMP-9
Primary lesion							
1	Nodular	+	+	+	f+	–	–
2	Nodular	+	+	+	±	–	–
3	Ulcer	+	+	±	–	–	–
4	Nodular	+	+	f+	–	–	–
5	Nodular	f+	+	f+	–	–	–
6	Nodular	+	+	f+	–	–	±
7	Macular	+	+	+	–	–	f+
8	Nodular	+	–	+	–	–	-
9	Macular	+	+	f+	–	–	f+
10	Macular	+	+	f+	–	–	-
11	Ulcer	+	+	f+	–	–	f+
12	Nodular	+	+	f+	–	–	–
13	Macular	+	+	±	–	–	–
14	Nodular	+	+	+	–	–	f+
15	Macular	+	+	+	–	–	f+
16	Macular	+	f+	f+	–	–	±
17	Macular	+	f+	f+	–	–	–
18	Nodular	ND	ND	ND	ND	ND	ND
19	Macular	+	+	+	–	–	–
20	Macular	+	+	+	–	–	f+
21	Nodular	f+	+	+	–	–	±
22	N/A	ND	ND	ND	ND	ND	ND
23	Nodular	f+	+	+	±	–	–
Lung metastatic lesion							
1	Cystic	+	+	±	–	–	–
	Nodular	+	+	f+	±	±	±
2	Cystic	+	f+	+	–	–	–
3	Cystic	+	+	+	±	–	–
	Nodular	+	f+	+	±	–	–
4	Cystic	–	–	f+	–	–	–
5	Cystic	f+	+	+	–	–	–
6	Cystic	+	+	–	±	–	–
7	Cystic	+	+	f+	–	–	f+
8	Cystic	+	+	+	–	–	–
9	Cystic	+	+	f+	–	–	–
10	Cystic	+	f+	+	–	–	±
11	Cystic	+	+	f+	–	±	–
12	Cystic	+	+	+	–	–	±
13	Nodular	+	+	f+	f+	–	f+
14	Nodular	+	+	+	–	–	±
15	Nodular	+	f+	+	+	±	f+
16	Nodular	+	+	f+	–	–	–
17	Nodular	+	+	+	–	–	–
18	Nodular	+	–	f+	–	–	f+
19	Perivascular	+	+	–	–	–	–
20	Perivascular	+	f+	+	–	–	–
21	Pleural	±	+	±	–	–	–
22	Pleural	+	+	+	–	–	–
23	Pleural	f+	+	f+	–	–	–
Liver metastatic lesion							
2	Cystic	+	+	f+	–	–	–
9	Nodular	–	–	–	–	–	–
12	Cystic	+	–	+	–	–	–
14	Nodular	f+	–	f+	–	–	–
22	Cystic	+	–	–	f+	–	–

Abbreviation: ND, not done

NOTE. Results of immunohistochemistry were represented as: (+) diffuse positive, (f+) focal positive, (±) equivocally stained, (–) negative.

Table 4 Results of immunohistochemical staining

	Primary lesion (n = 21)		Lung metastasis (n = 25)		Liver metastasis (n = 5)		Capillary hemangioma (n = 10)		Carvenous hemangioma (n = 10)	
	+	–	+	–	+	–	+	–	+	–
D2-40	21	0	23	2	4	1	0	10	0	10
ERG	20	1	23	2	1	4	10	0	10	0
MMP-1	20	1	21	4	3	2	5	5	1	9
MMP-2	1	20	1	24	0	5	0	10	0	10
MMP-7	0	21	0	25	0	5	0	10	0	10
MMP-9	7	14	4	21	0	5	0	10	1	9

NOTE. Results of immunohistochemistry were represented as: (+) diffuse or focal positive, (–) equivocally stained or negative.

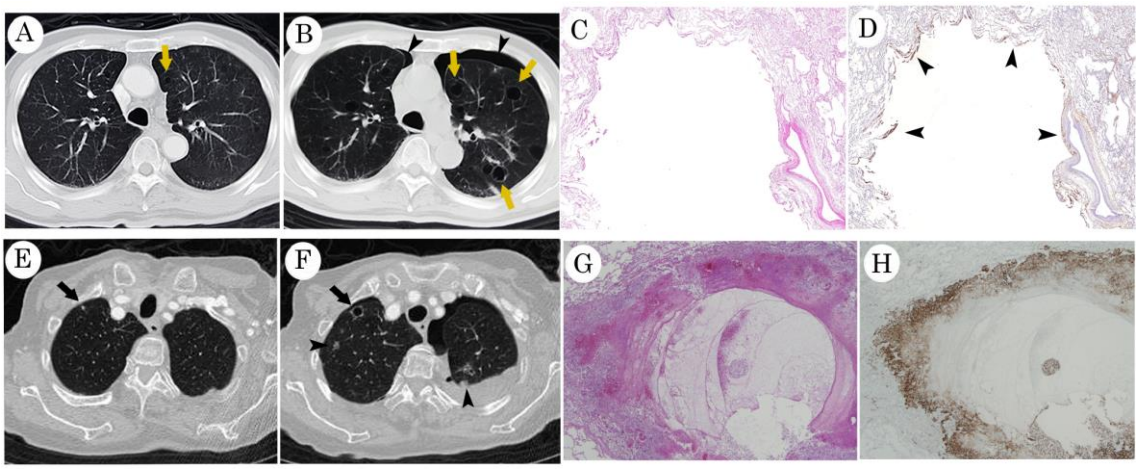

Fig. 1　A, A lung CT image of Case 2 at the early stage. A small thin-walled cyst is observed in the left lung (arrow). B, CT image after 6 months. There are multiple thin-walled cysts throughout the lung (arrows) with pneumothorax (arrow heads). C, Cystic metastasis of the lung in which it is difficult to identify the tumor cells (Hematoxylin and eosin [HE] staining). D, D2-40 immunostaining reveals positive tumor cells at the luminal surface as a thin layer (arrow heads) (submacroscopic photographs, C and D). This is a common pattern of cystic pulmonary metastasis in this study. E, A lung CT image of Case 1 on early stage. A tiny nodular lesion is observed in the right lung, upper lobe (arrow). F, CT image after 6 weeks. The tiny nodule turned into a thin-walled cyst (arrow). Two small irregular nodular shadows are also observed (arrow heads). Pneumothorax and pleural fluid are seen in the left pleural cavity. G, A cystic metastatic lesion of the lung. Proliferating tumor cells surround the central hemorrhagic and necrotic part (HE staining). H, Tumor cells were clearly stained for D2-40 (original magnification ×25, G and H).

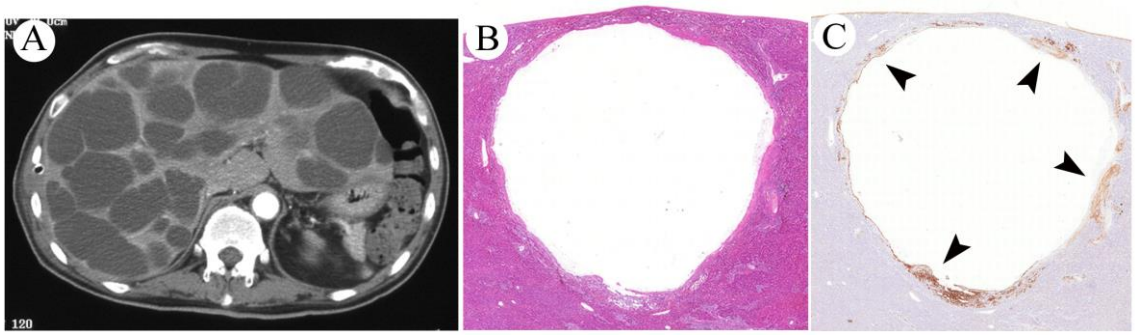

Fig. 2　A, CT image of the liver revealing many cystic metastatic lesions. B, Cystic metastasis of the liver. The tumor cells are difficult to identify with HE staining. C, D2-40 staining revealed D2-40-positive tumor cells at the luminal surface (arrows) (submacroscopic photographs, B and C).

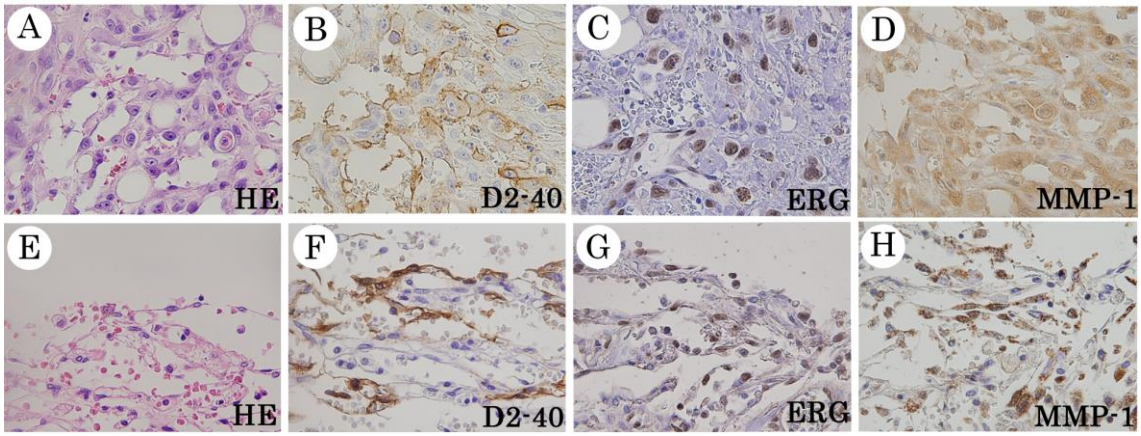

Fig. 3　A, Primary lesion of the scalp. Pleomorphic cells proliferation with irregular capillary-like structures (HE staining). B, The pleomorphic cells are strongly stained D2-40 immunopositive, and are considered tumor cells. C, The tumor cells show nuclear staining for ERG. D, MMP-1 staining. MMP-1 is positive in the tumor cells. E, The wall of the pulmonary cystic metastasis. It is difficult to identify tumor cells (HE staining). F, D2-40 staining of the same site in E. Spindle-shaped tumor cells are immunopositive for D2-40. G, ERG is strongly positive in the nuclei of tumor cells. H, MMP-1 is strongly expressed in the tumor cells (original magnification ×400, A-H).

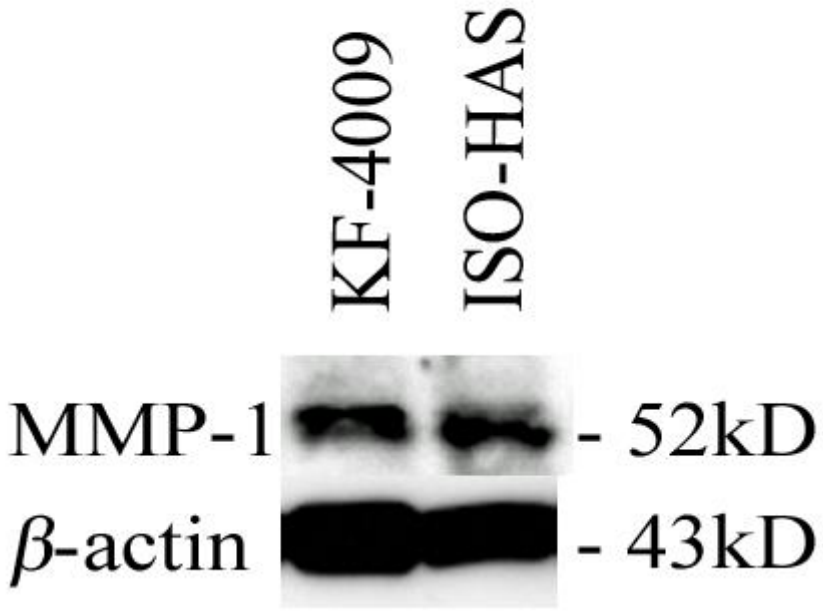

Fig. 4　MMP-1 expression in the human hemangiosarcoma cell line (ISO-HAS). The MMP-1 expression was examined by western blot analysis in the ISO-HAS cell line. The normal human fibroblast cell line (KF-4009) was used as a positive control for the MMP-1 expression.