Histological and immunohistochemical prognostic factors of primary angiosarcoma

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The authors declare that we have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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Abstract

Angiosarcoma is a malignant vascular endothelial neoplasm with various histological patterns. Despite its highly malignant potential, histological prognostic prediction has not been adopted for angiosarcoma. This study aimed to establish a method of predicting the prognosis of primary angiosarcoma. Formalin-fixed, paraffin-embedded samples from 104 primary angiosarcomas were prepared. All the cases were reviewed based on histological examinations with H&E staining. Because the French Fédération Nationale des Centres de Lutte Contre Le Cancer system (FNCLCC) is not adopted for angiosarcoma, we experimentally established a modified version of FNCLCC. Immunohistochemical staining for ERG, CD31, CD34, D2-40, HHV-8, p16, C-MYC, and p53 was performed. Fluorescence in situ hybridization (FISH) was performed for 31 cases to assay *c-MYC* gene amplification. Multivariate analysis revealed that age (>70 years old) ($p = 0.0011$), non-cutaneous angiosarcoma ($p = 0.0265$), metastasis on diagnosis ($p < 0.0001$), size ≥5cm ($p = 0.0388$), no taxane chemotherapy ($p = 0.0388$), strong nuclear atypia ($p = 0.0087$), and the presence of luminal structure in ≥50% of the tumor volume ($p = 0.0009$) were independent poor prognostic factors. Among angiosarcomas with luminal formation, mFNCLCC scores were significantly correlated with a poorer prognosis. The overexpression of p16 was associated with less luminal formation ($p = 0.0192$). Immunohistochemical analysis of C-MYC showed a moderate level of concordance with FISH (Kappa value = 0.45). This study suggested that luminal formation and nuclear atypia may be poor histological

prognostic factors of angiosarcoma and that mFNCLCC would be useful for predicting the prognosis of angiosarcoma with luminal formation.

(249 words)

Keywords: angiosarcoma, histology, p16, MYC

Introduction

Angiosarcoma is a rare malignant mesenchymal tumor defined as a “highly malignant neoplasm with endothelial differentiation” in the WHO classification [1,2]. Angiosarcoma accounts for 2% of all soft-tissue sarcomas and 5.4% of cutaneous soft-tissue sarcomas [3]. Histologically, angiosarcoma is composed of neoplastic endothelial cells, showing irregular vessels, intracytoplasmic vacuoles, and hemorrhage.

Regarding its histological variation, some morphologic patterns of angiosarcoma have been identified, such as “epithelioid feature,” [4], and “solid pattern” [5]. Malignant tumors usually show a worse prognosis if they show less differentiation, abundant mitosis, and/or necrosis. However, in angiosarcoma, it is not recommended that these variables be used to evaluate malignancy [6]. There has been controversy regarding the grading of angiosarcoma. Histological gradings, epithelioid feature, solid pattern, vascular formation, MIB-1 index, and necrosis had been stated to be prognostic factors of angiosarcoma by different authors [4, 5, 7-10].

Gene mutation analysis of angiosarcoma is now underway. It has been shown that *c-MYC* gene amplification occurs in most cases of secondary angiosarcoma, although its significance in tumorigenesis is unclear [11]. Meanwhile, the gene alterations that drive primary angiosarcoma also remain unclear. Although some primary angiosarcoma-related genetic mutations have been reported, none of them is related to prognosis or specific histological patterns [11]. In this study, we aimed to evaluate histological characteristics and immunohistochemical profiles of angiosarcoma to reveal the histological background of

the aggressive course of angiosarcoma and to establish its histological prognostic factors.

Materials and Methods

Materials

This study was conducted in accordance with the principles embodied in the Declaration of Helsinki. This study was also approved by the Ethics Committee of Kyushu University (Nos. 29-625, 29-429, 2020-361, 2021-165). One hundred and eleven cases, previously diagnosed with angiosarcoma, were retrieved from the soft-tissue tumors registered in the files of the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, between 1984 and 2020. Secondary angiosarcomas arising after radiation therapy or long-term lymphedema were excluded. Formalin-fixed, paraffin-embedded samples of angiosarcomas from 104 primary tumors of 104 patients were prepared for immunohistochemical study.

Hematoxylin and eosin (H&E)-stained slides were reviewed by two of the authors (TI and YY) to confirm the original diagnosis, and a single representative tumor block was chosen for immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) analysis. Histologically, all available cases were included in the histological spectrum of angiosarcoma described in the WHO classification [2]. The diagnosis of each angiosarcoma case was confirmed by the histological characteristics of endothelial differentiation, such as irregular vessels, intravascular components, and/or

intracytoplasmic vacuoles, and IHC for endothelial markers. All cases were IHC-positive for at least two of the endothelial markers. Kaposi sarcoma was ruled out by immunohistochemical negativity for HHV-8 antibody.

Clinicopathological data

Clinicopathological data such as age, sex, tumor size, tumor location, presence of metastasis, and selection of treatments were retrieved. Medical information on the clinical course was available in 68 cases. The length of follow-up was 28.6 months on average. All the specimens used for histological and immunohistochemical examinations were sampled prior to the treatment.

Histopathological examination

All the cases were reviewed based on histological examinations using H&E staining. The number of biopsied specimens included in this study was 75 and that of resected specimens was 29.

The following histopathological findings were evaluated: nuclear atypia, nucleoli, luminal structure, epithelioid feature, conventional solid growth pattern, mitotic count, necrosis, and karyorrhexis. Among the evaluated histological findings, the reproducibility rates of nuclear atypia, nucleoli, luminal structure, epithelioid feature, and conventional solid growth pattern were evaluated using the Kappa statistic by two of the authors (TI and YY).

The definition of each histological finding was summarized in Supplementary Table 1. Nuclear atypia was scored as follows: 1: small, flat, and spindle-shaped nuclei as in normal endothelial cells, 2: intermediate between scores 1 and 3, and 3: large, hyperchromatic, and round or bizarre nuclei. Nucleoli were scored as follows: 1: barely visible or not recognizable, 2: intermediate between scores 1 and 3, and 3: large prominent nucleoli. The luminal structure was defined as the formation of vessels lined by neoplastic endothelial cells or the formation of clefts containing erythrocytes within tumor cell nests. We have measured the % of the area of luminal formation by neoplastic cells. We defined "angiosarcoma with luminal formation" (ASLF) if luminal structure composes more % of the area than the threshold, and "angiosarcoma with less luminal formation" (ASLLF) if not. The threshold of 30%, 50% and, 80% were separately established. "Angiosarcoma with epithelioid feature" was defined as angiosarcoma with at least a focal area of round to polygonal angiosarcoma cells with enlarged nuclei, prominent nucleoli, and abundant cytoplasm in accordance with the previous report [4]. "Conventional solid pattern" was defined as angiosarcoma composed of a dense proliferation of tumor cells with little or no stroma as well as no formation of tubular structures in more than 80% of the area of the lesion in accordance with the previous report [5]. Karyorrhexis was defined for cells undergoing apoptosis, with eosinophilic cytoplasm and condensed nuclei.

Because the American Joint Committee on Cancer (AJCC) staging system 8th edition [6] indicates that the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) scoring system is not

applicable to angiosarcoma, we experimentally adopted this system to evaluate the prognosis of angiosarcoma. We have adopted nuclear atypia instead of histological differentiation and named it modified FNCLCC (mFNCLCC). mFNCLCC scoring system is evaluated as follows: nuclear atypia (score 1–3) + necrosis (score 0–2) + mitosis (score 1–3). The total score thus ranged from 2 to 8 points (Supplementary Table 1). When there was a mixture of different histological patterns in a tumor, the most extensive pattern was adopted.

Immunohistochemistry

Immunohistochemical staining was performed for all available cases for which a sufficient quantity of specimens was available. Formalin-fixed, paraffin-embedded tissue was sectioned at 3 μm thickness. The primary antibodies, their dilutions, and the antigen retrieval are summarized in Supplementary Table 2. The immune complexes were detected with the DAKO EnVision Detection System (Dako, Glostrup, Denmark). Immunohistochemical positivity of each antibody was judged in accordance with the following thresholds: ERG, CD31, CD34, D2-40, and HHV-8 were judged as positive if more than 10% of the tumor cells were positively stained, while for p16 the threshold was ≥70% [12] and for p53 ≥20% [13]. Meanwhile, the threshold of c-MYC was based on ≥10% of the tumor cell positivity and at the same time based on the intensity of the staining. The intensity was judged by comparison to the internal positive control (epidermis) or by visually confirming the whole nuclei to be stained in dark brown [14].

These thresholds were decided in accordance with previous studies.

Fluorescence in situ hybridization (FISH)

To assay amplification of the *c-MYC* gene, dual-color FISH was performed for 31 cases of angiosarcoma for which sufficient specimens resected from 2010 to 2020 were available, using ready-made FISH probes (GSP Laboratories, Kawasaki, Japan). Paraffin-embedded tissue was sectioned at 3 μm thickness and processed in accordance with the manufacturer's instructions. The *c-MYC* gene was labeled with red signals and *CEN8p* with green. Fifty nuclei were counted to estimate the proportion of tumor cells with amplified *c-MYC* genes. *c-MYC* amplification was defined as a ratio of *c-MYC*/*CEN8p* ≥2. Polysomy was defined as a gain of both *c-MYC* and *CEN8p*, and a ratio of *c-MYC*/*CEN8p* <2.

Statistical analysis

Using Kaplan–Meier's method, the relationships between overall survival (OS) and clinicopathological, histological, and immunohistochemical parameters were assessed by the log-rank test. As for disease-specific survival (DSS) and metastasis-free survival (MFS), the same relationships were assessed by the same method. The numbers of cases available for prognostic analysis were 68, 68, and 57 for OS, DSS, and MFS, respectively. The same relationships were assessed by the same method exclusively for 24 resected angiosarcomas. Multivariate analysis was performed by the Cox proportional hazard model

using the backward selection method. We included all candidates for prognostic factors, which included age, metastasis, primary site, taxane therapy, luminal structure (≥30%), luminal structure (≥50%), necrosis, and previously reported prognostic factors, namely, sex, size, resection of the primary lesion, radiation therapy, mitosis, nuclear atypia, conventional solid pattern, epithelioid feature, and MIB-1 labeling index. Fisher's exact test was used to evaluate the significance of the relationships between ASLLF or ASLF and each parameter described above. Thus, all the clinicopathological, histological, and immunohistochemical findings were analyzed for their correlations to the luminal structure using Fisher's exact test. A two-sided p-value of <0.05 was considered statistically significant. All statistical analyses were conducted with the JMP statistical software package (15.1.0).

Results

Clinical results

The clinicopathological characteristics are shown in Table 1. The mean age of the patients at diagnosis was 68.9 years old [standard deviation (SD) 15.0, range 7–92]. The male/female ratio was 1.67 (65 males and 39 females). The origin of the tumor was skin in 67 cases (head and neck in 60 cases, extremity 5 cases, trunk 2 cases), bone and soft tissue in 13 cases (soft tissue of extremity 7 cases, soft tissue of trunk 2 cases, femur 2 cases, mandible 1 case, spine 1 case), liver and spleen in 12 cases (liver 7 cases, spleen 5 cases), mediastinum in 8 cases (heart 3 cases, aorta 1 case, thoracic wall 4 cases), and others

in 4 cases (cecum 1 case, duodenum 2 cases, adrenal gland 1 case). As for the treatment, among 59 cases for which complete medical records were available, 15 cases underwent resection of the lesion, 41 cases received radiation therapy, and 28 cases underwent chemotherapy using taxane. The survival data of 68 available cases revealed a 5-year survival rate of 21.7% and 42 tumor-related deaths (61.8%).

Histological results

The results of the histological review are shown in Table 2. Among 104 cases, nuclear atypia scores of 1, 2, and 3 were seen in 27 (26.0%) (Figure 1a), 40 (38.5%) (Figure 1b), and 37 cases (35.6%) (Figure 1c), respectively. Nucleoli scores of 1, 2, and 3 were seen in 36 (34.6%) (Figure 1d), 34 (32.7%) (Figure 1e), and 34 cases (32.7%) (Figure 1f), respectively. The luminal structure was ≥30% in 86 cases (82.7%) (Figure 1g and h), <30% in 18 (17.3%) (Figure 1i), ≥50% in 79 (76.0%), <50% in 25 (24.0%), ≥80% in 74 (71.2%), and <80% in 30 (28.8%). The conventional solid pattern was seen in 20 cases (19.2%) (Figure 1j) and the epithelioid feature in 25 (24.0%) (Figure 1k). Mitosis scores of 1, 2, and 3 were seen in 79 (76.0%), 15 (14.4%), and 10 cases (9.6%), respectively. Karyorrhexis was found in 63 cases (60.6%). Finally, the kappa values of nuclear atypia, nucleoli, luminal structure, conventional solid pattern, and epithelioid feature were 0.798, 0.783, 0.849, 0.799, and 0.800, respectively.

Immunohistochemical results

The results of the immunohistochemical analysis are shown in Table 2. ERG was positive in all cases (100%) (Figure 2a). CD31 was positive in 91 cases (95.8%) (Figure 2b), CD34 in 70 cases (72.2%) (Figure 2c), and D2-40 in 65 cases (70.7%) (Figure 2d). MIB-1 index was 25% on average (SD 0.266), with a median of 10%, and cases with an MIB-1 index of ≥25% accounted for 39.1% (36 cases). c-MYC was positively stained in 19 cases (21.6%) (Figure 2e), p16 in 17 cases (18.9%) (Figure 2f), and p53 in 50 cases (61.0%) (Figure 2g).

Fluorescence in situ hybridization (FISH)

Among the 31 cases for which FISH study was performed, 4 cases showed *c-MYC* amplification and 27 did not (Figure 2h). Within this latter group, 2 cases showed polysomy of *c-MYC* (Figure 2i). Among 7 cases positive for c-MYC on IHC, 3 showed amplification on FISH and 2 shows polysomy on FISH. Among 24 cases negative for c-MYC on IHC, 23 showed no amplification nor polysomy on FISH and 1 showed amplification on FISH. FISH analysis of *c-MYC* amplification showed a moderate correlation to the results of immunohistochemical staining (Kappa value 0.45, SD 0.199, concordance rate 84%).

Survival analysis

Among clinical features, younger age (≤70 years old) (Figure 3a, $p = 0.0040$), skin as the primary disease site (Figure 3b, $p = 0.0262$), no metastasis (Figure 3d, $P < 0.0001$), and taxane chemotherapy ($p =$

0.0021) were proven to be favorable prognostic factors in terms of DSS (Table 1). Among histological features, luminal structure <30% (p = 0.0399), luminal structure <50% (Figure 3c, p = 0.0330), and no necrosis (p = 0.0175) were suggested to be associated with a better DSS-based prognosis (Table 2). Among these features, cases without necrosis showed significantly better MFS (p = 0.0169), and luminal structure <30% and <50% showed tendencies for better MFS (p = 0.0826 and 0.0893, respectively). In immunohistochemistry, no factors showed a significant association with a better prognosis in terms of DSS; however, cases positive for p16 showed a tendency for a better DSS-based prognosis (p = 0.0636) and significantly better MFS (p = 0.0312).

The univariate survival analysis on 24 resected cases revealed that younger age (p = 0.0937), skin as the primary disease site (p = 0.0945), luminal structure (p = 0.0983) had a tendency for a better prognosis (supplementary data 3).

Multivariate analysis

Multivariate analysis on DSS indicated that age (p = 0.0011), primary site (p = 0.0265), metastasis (p <0.0001), size (p = 0.0127), Taxane chemotherapy (p = 0.0388), nuclear atypia (p = 0.0087), and luminal structure (≥50%) (p = 0.0009) were independent prognostic factors. Among the histological features, nuclear atypia and luminal structure (≥50%) showed independent relationships with DSS (p = 0.0087 and 0.0009, Hazard ratio 3.23 and 6.08, 95% CI 1.35-7.74 and 2.09–17.7, respectively)

(Supplementary data 4).

Statistical comparison between ASLLF and ASLF

Fisher's exact test was utilized. Upon comparing ASLLF and ASLF, the following clinical, histopathological and immunohistochemical characteristics were identified (Supplementary data 5). At the threshold of 50%, compared with ASLF, ASLLF had significantly higher mitotic activity [>10/10 high-power fields, p = 0008, odds ratio (OR) 6.05], more karyorrhexis (p = 0.0089, OR 4.63), higher rate of nuclear atypia (p <0.0001, OR 10.73), and more prominent nucleoli (p = 0.0004, OR 6.02), more positive rate for CD34 (p = 0.0027, OR 4.8) and more for p16 (p = 0.0145, OR 5.36). At the threshold of 30% and 80%, the results were similar to that at 50% (supplementary data 5).

mFNCLCC scoring system

No statistically significant relationship between mFNCLCC score and DSS-based prognosis was evident when all cases were analyzed (Figure 3e). However, when only ASLF cases were analyzed, mFNCLCC score and prognosis were significantly correlated. A low mFNCLCC score (2–3) was associated with a significantly better prognosis in terms of DSS (p = 0.0011) among ASLF cases. As for ASLLF, no significant prognostic difference was seen between those with low (2–3) and high (4–8) mFNCLCC scores

(p = 0.4098). The DSS-based prognosis of three groups (ASLLF, ASLF with low mFNCLCC score, and ASLF with high mFNCLCC score) is shown in Figure 3f. As for MFS-based prognosis, ASLLF with a low mFNCLCC score was associated with a significantly better prognosis (p = 0.008) compared to ASLLF with a high mFNCLCC score (supplementary data 6). Moreover, among ASLLF cases with a low mFNCLCC score, no distant metastasis was seen (observation period 9.0–85.4 months, 38.9 months on average).

Analysis of the 24 resected cases revealed that compared to ASLF with high mFNCLCC scores, ASLF with low mFNCLCC scores showed better DSS-based prognosis (p = 0.0179) (supplementary data 3).

Discussion

Since angiosarcoma was first mentioned in the seventh edition of the AJCC staging system published in 2010, this disease has been not recommended to be graded under its histological grading system, either by AJCC or by the UICC TNM Classification [15], given that it is an aggressive tumor regardless of its histological differentiation. In previous studies, several factors such as epithelioid features had been stated to be prognostic by different authors, but it has been controversial whether these histological features have true prognostic value [4, 5, 7-10]. In the present study, luminal structure ≥50% and strong nuclear atypia were indicated to be significant unfavorable prognostic factors. This result was compatible with the previous report by Detternborn and Sinnamon [5, 8].

We considered that evaluation of luminal structure and nuclear atypia may be valuable for predicting the prognosis of angiosarcoma. As for the threshold of luminal structure, at 50% the result showed the lowest p-value in the univariate analysis and was detected as an independent prognostic factor by the multivariate analysis. These data suggested ASLLF's prognosis was distinctive when distinguished from ASLF according to the threshold of 50%.

Some researchers reported that luminal formation in angiosarcoma was a prognostic factor associated with a favorable prognosis, in which well-differentiated angiosarcomas with clear differentiation into endothelial cells were extracted as vasoformative, concluding that those angiosarcomas have less cytological atypia and better prognosis [9, 10]. In the present study, we defined luminal formation as the "formation of vessels or clefts" because, in angiosarcoma, if clefts containing red blood cells are found among proliferated atypical cells, it is difficult to differentiate those clefts from irregular vessels. Finally, we showed that luminal formation was a statistically independent prognostic factor of unfavorable clinical course. In addition, luminal formation presented a statistical tendency for distant metastasis. From the above, we considered luminal structure may be an unfavorable prognostic factor if both formations of vessels and clefts are regarded as luminal structures.

The mechanism of luminal formation to contribute to unfavorable prognosis remains unclear, however, we speculated that angiosarcoma may develop hematogenous metastasis by possession of connection to the systemic circulation through the luminal structure. On the other hand, nuclear atypia was

also detected as a prognostic factor in the current study. Considering the above, the prognosis of angiosarcoma may be dependent on the balance between metastasizing potential from luminal formation and proliferative activity. Angiosarcomas with low-grade nuclear atypia and clear luminal formation may have a better prognosis because of low-grade nuclear atypia despite luminal formation, while angiosarcomas with high-grade nuclear atypia and less luminal formation may have less metastatic potential despite its high proliferative potential.

In this paper, we proposed using an mFNCLCC grading system for accurate prediction of the prognosis of angiosarcoma and demonstrated that angiosarcomas fall within the remit of mFNCLCC when ASLLF is excluded from the analysis. As the original FNCLCC system does not include nuclear atypia in its criteria, histological differentiation of angiosarcomas is only scored as 2 or 3 in the original FNCLCC. We suggest that markers for cell proliferation such as nuclear atypia and mitosis could be of value in prognosis after classifying angiosarcomas into ASLF or ASLLF.

As for the genetics of primary angiosarcoma, mutations in components of the *MAPK* pathway, *TP53* and *c-MYC*, the presence of the fusion gene *NLP160-SLC43A3*, and increased tumor mutation burden have been reported [16-18]. However, no reports have revealed the relationship between mutations of those genes and the histological pattern. The present study demonstrated that the overexpression of p16 was significantly correlated with the histological pattern of less tubulization and less metastatic potential of angiosarcoma. We suggest that p16 overexpression may be associated with the less tubular histology and

biological behavior of angiosarcoma. However, there is a need to reveal the molecular background of p16 overexpression in angiosarcoma.

p16 is a regulatory protein of the cell cycle, while also being related to cell senescence and angiogenesis [19, 20]. It was reported that, when p16 is overexpressed after Rb protein inactivation, it binds to HIF-1α, a transcription factor that targets the VEGF promoter, thereby reducing VEGF production [21]. Through VEGF signal transduction, endothelial cell differentiation and migration are promoted [22]. In ASLLF, luminal formation may be inhibited by p16 overexpression, as a result of reduced expression of VEGF. However, *p16* is also related to telomere shortening and cell cycle arrest through a different pathway from angiogenesis. *p16*, as a multifunctional gene, may be important to understanding angiogenesis and tumorigenesis in angiosarcoma.

To date, no definitive criteria have been established for defining *c-MYC* positivity in immunohistochemical analysis; instead, a variety of criteria have been adopted [14, 23, 24]. To ensure the accuracy of the immunohistochemical test of c-MYC, we evaluated the concordance of c-MYC IHC and *c-MYC* FISH. Meanwhile, *c-MYC* was not shown to be significant for the prognosis of DSS. Although primary angiosarcomas may feature *c-MYC* amplification or polysomy, this genetic change may not impact the tumor behavior.

There are some limitations in this study. Firstly, this study includes biopsied specimens in the analysis since not many angiosarcoma patients can undergo surgery and it is difficult to survey histological

analysis of only resected specimens. However, the results of analysis exclusively using resected specimens were comparable to the results including biopsied specimens, although the difference did not reach statistical significance possibly due to the small number of cases. Accumulation of resected specimens and a larger study containing only resected angiosarcoma are expected. Second, it was disappointing that we could not extract molecular biological data. However, the endpoint of the current investigation is to reveal the relationship between histology and prognosis. It is desired that genetic data will be presented in the following research about the molecular characteristics of angiosarcoma. Thirdly, we cannot apply our findings to primary breast angiosarcomas. Primary breast angiosarcomas have distinctive characteristics. They occur in younger women and tend to show so well-differentiated lesions that may occasionally pose difficulty in differentiating from benign hemangioma. Moreover, mutations in *KDR(VEGFR-2)* and *PIK3A* are found more frequently in them [25]. Because of these clinical, pathological, and genetic differences, the primary breast angiosarcoma cases were excluded from the current investigation. Primary breast angiosarcomas have been adapted to a 3-tier histological grading system (or 2-tier according to the latest study) [26-28]. However, we considered that it is controversial that these systems can be adopted for the other angiosarcomas. The enrolled patients were not enough for location-specific analysis. We need an accumulation of cases to clarify the differences among angiosarcomas from different locations.

In conclusion, this study suggested that luminal formation may be related to the high metastatic potential of angiosarcoma and that mFNCLCC would be useful for predicting the prognosis of lumen-

forming angiosarcoma. Also, p16 overexpression may be associated with luminal formation in angiosarcoma.

Declarations

Compliance with Ethical Standards

This study was conducted in accordance with the principles embodied in the Declaration of Helsinki. This study was also approved by the Ethics Committee of Kyushu University (Nos. 29-625, 29-429, 2020-361, 2021-165).

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Authors' contributions

T. Ichiki performed the research and wrote the paper. Y. Yamada and T. Ito contributed to the research design and slide review. Y. Nakashima, M. Nakamura, T. Yoshizumi, A. Shiose, and K. Akashi contributed to the acquisition of the samples. T. Nakahara and Y. Oda designed the research and gave final approval of the manuscript. All authors critically reviewed and approved the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest to disclose.

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Figure legends

Figure 1. a–c: Histological evaluation of nuclear atypia (a. score 1, b. score 2, c. score 3). d–f: Histological evaluation of nucleoli (d. score 1, e. score 2, f. score 3). g-i: Histological evaluation of luminal structure (g: formation of vessels lined by endothelial-like cells, h: formation of clefts containing erythrocytes, i: No formation of vessels or clefts). j: conventional solid pattern, k. epithelioid feature.

Figure 2. a–g: Examples of cases positive for immunohistochemical staining (a. ERG, b. CD31, c. CD34, d. D2-40, e. c-MYC, f. p53, g. p16). h and i: Fluorescence in situ hybridization for *c-MYC*. The red signal stands for *c-MYC* and green for *CEN8p*. (h. Amplification was suggested if the red/green ratio was ≥2. i. Polysomy was defined as a gain of both *c-MYC* and *CEN8p*, and a ratio of *c-MYC*/*CEN8p* <2.)

Figure 3. Kaplan-Meier's curves of disease-specific survival (a. age, b. primary site of angiosarcoma, c. luminal structure, d. metastasis on diagnosis, e. mFNCLCC score, f. angiosarcomas with less luminal formation (ASLLF) and angiosarcomas with luminal formation (ASLF) in different mFNCLCC scoring groups).

Legends for supplementary data.

Supplementary data 1. Definitions of histological characters in histological evaluation.

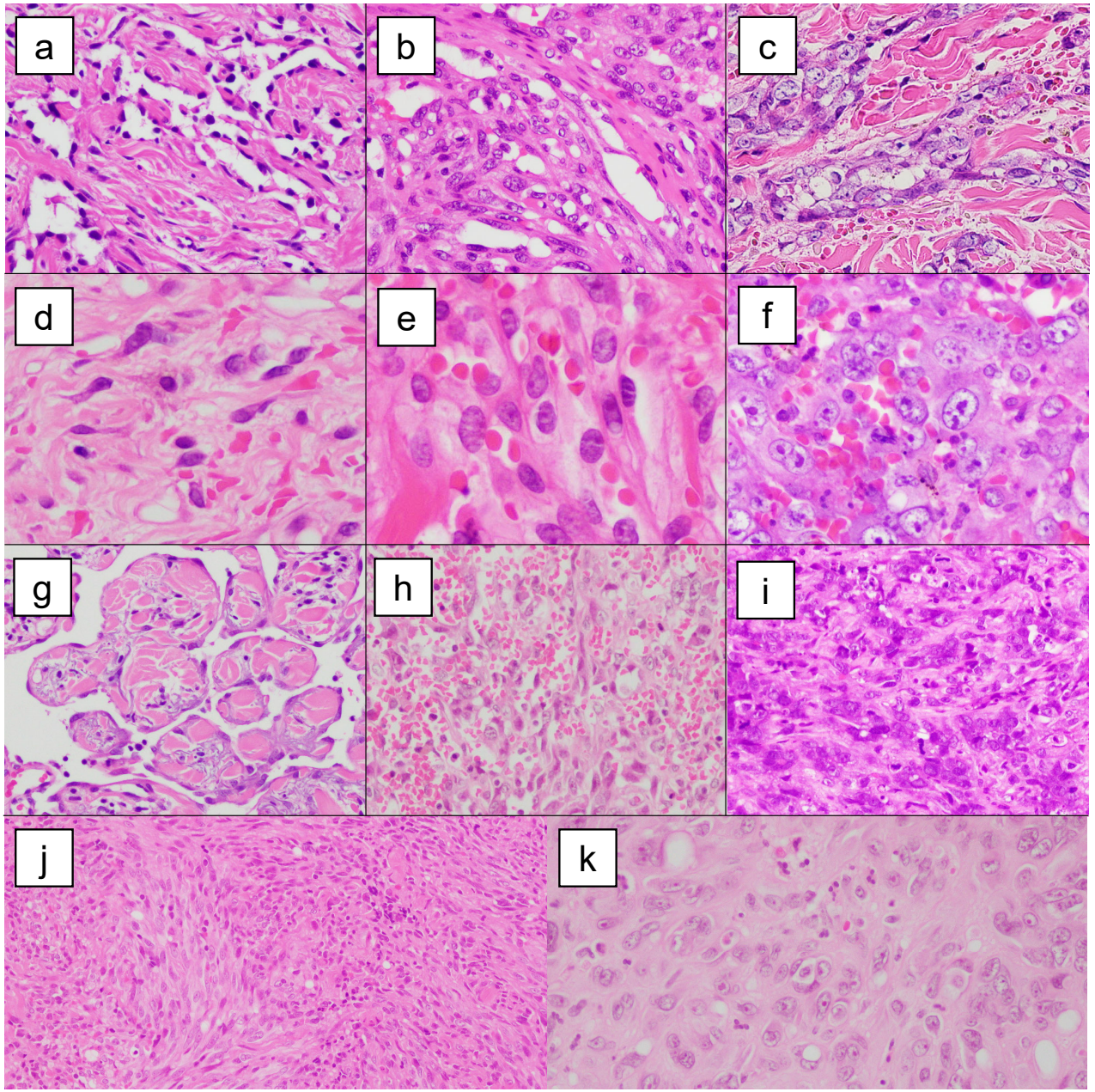
Supplementary data 2. Antibodies used for immunohistochemistry.

Supplementary data 3. Kaplan-Meier's curves of disease-specific survival of resected cases (a. age, b. primary site of angiosarcoma, c. luminal structure, d. mFNCLCC score, e. angiosarcomas with less luminal formation (ASLLF) and angiosarcomas with luminal formation (ASLF) in different mFNCLCC scoring groups).

Supplementary data 4. Multivariate proportional hazard analysis on disease-specific survival (DSS).

Supplementary data 5. Relationship between luminal structure and other clinical and histological features.

Supplementary data 6. Kaplan-Meier's curve of MFS-based prognosis of ASLLF with high and low mFNCLCC score.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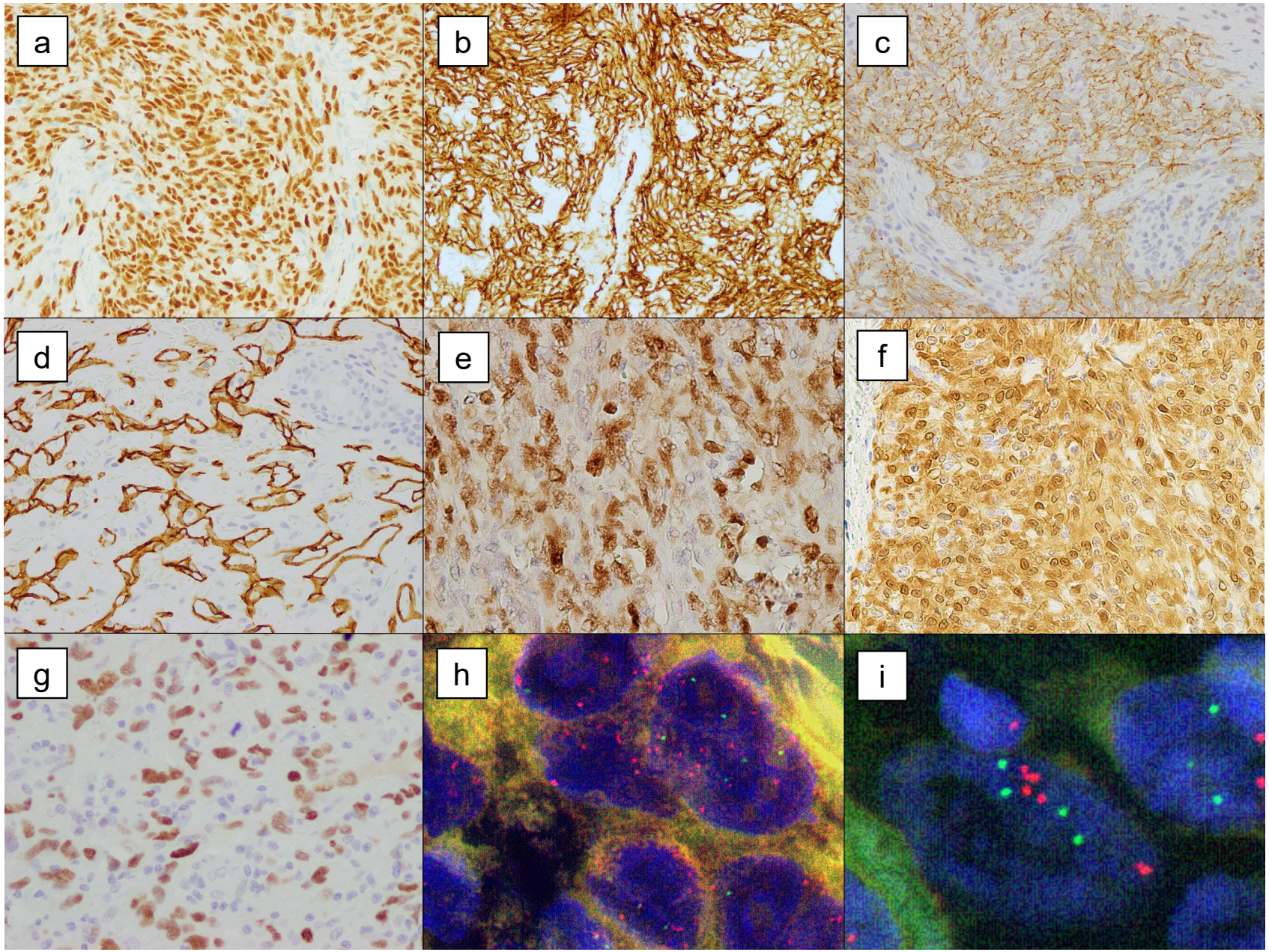
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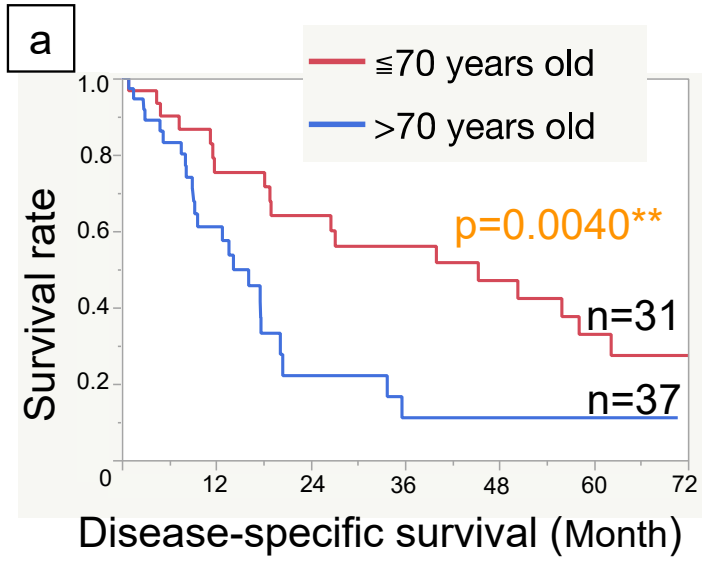
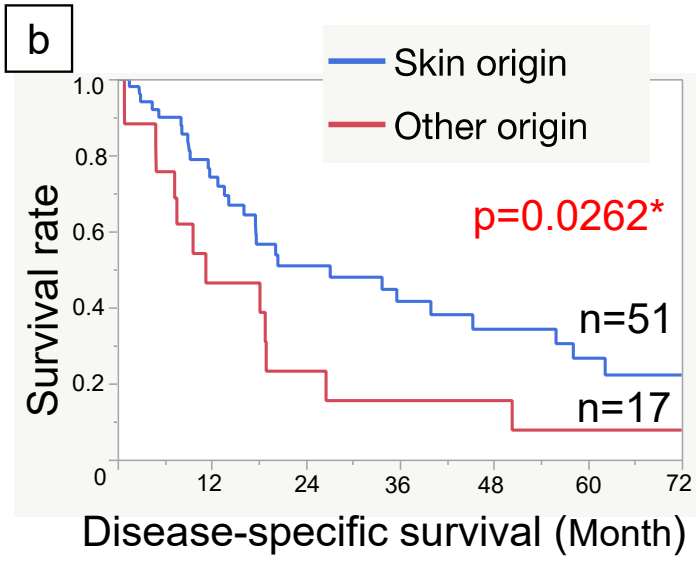
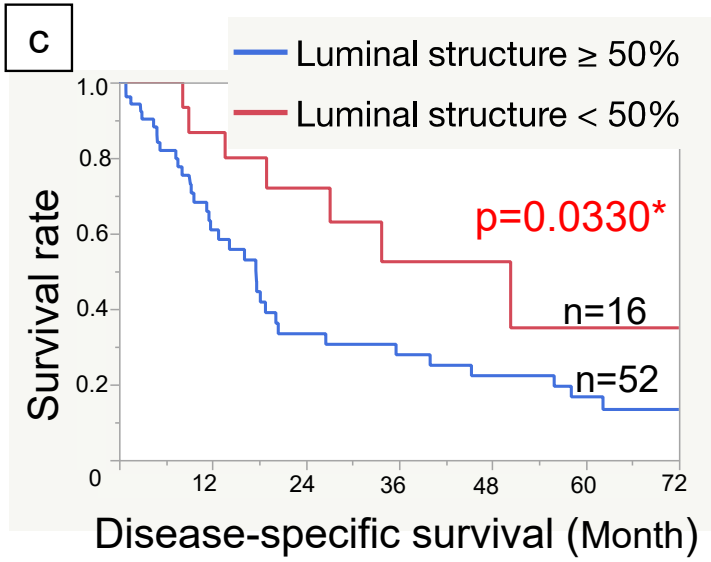
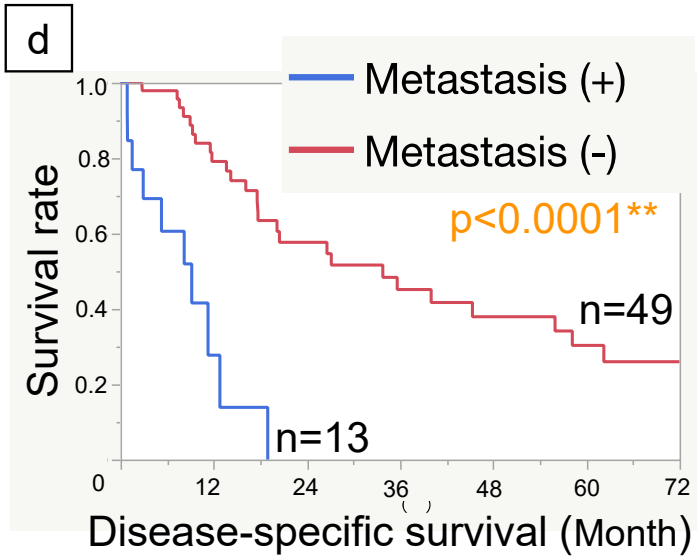
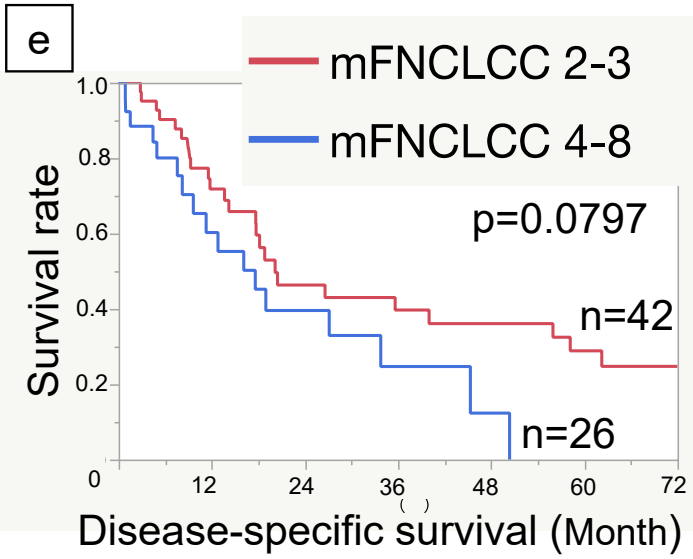
Kaplan-Meier's curve

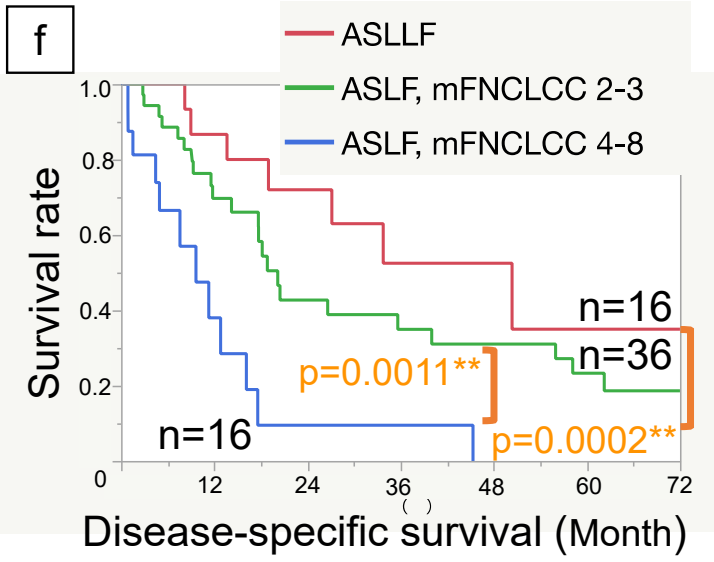












Supplementary Table 1. Definitions of histological characters in histological evaluation

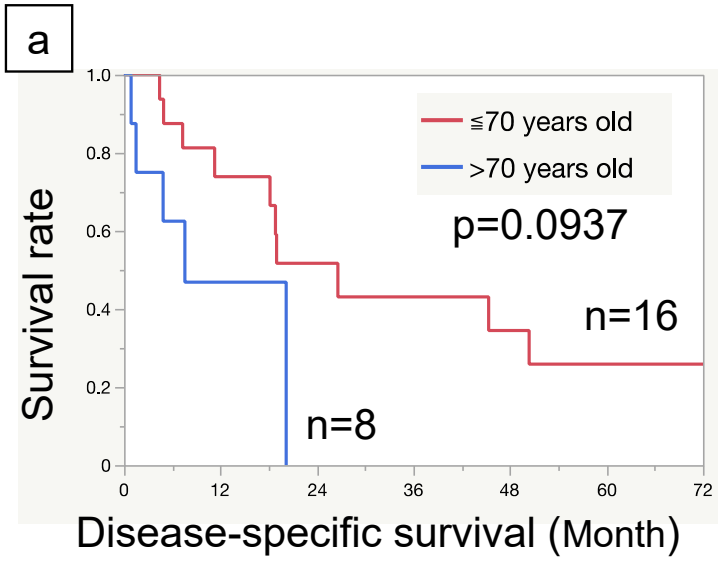
Histological character	Strata	Definition
Nuclear atypia score	1	Small, flat and spindle nuclear as normal endothelial cells
	2	Intermediate between 1 and 3
	3	Large, hyperchromatic, and round or bizarre nuclear
Nucleoli score	1	Nucleoli barely seen or not recognizable
	2	Intermediate between 1 and 3
	3	Large prominent nucleoli
Mitosis score	1	Less than 10 mitotic figures/10HPF
	2	10 to 19 mitotic figures/10HPF
	3	More than 19 mitotic figures/10HPF
Necrosis score	0	No necrosis
	1	Necrosis in less than 50% area of the lesion
	2	Necrosis in 50% or more area of the lesion
mFNCLCC score	2	
	3	
	4	
	5	Nuclear atypia score (1-3) + Necrosis score (0-2) + Mitosis score (1-3)
	6	
	7	
	8	

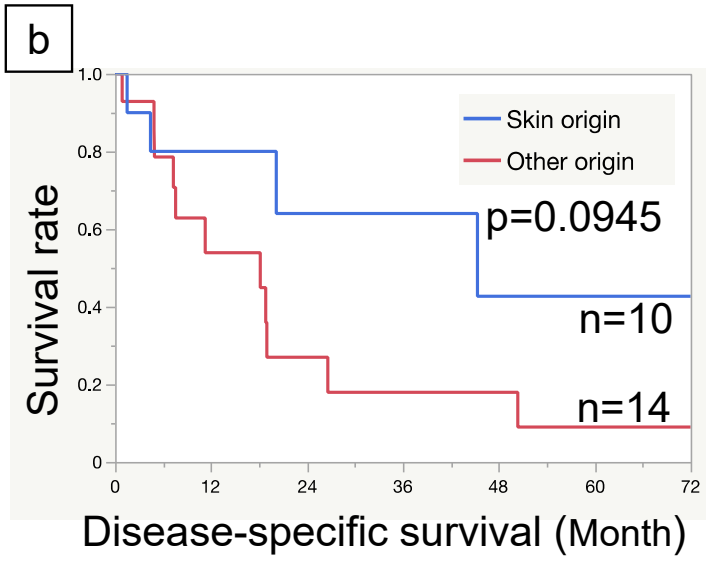
HPF indicates high power field. mFNCLCC indicates modified French Fédération Nationale des Centres de Lutte Contre Le Cancer.

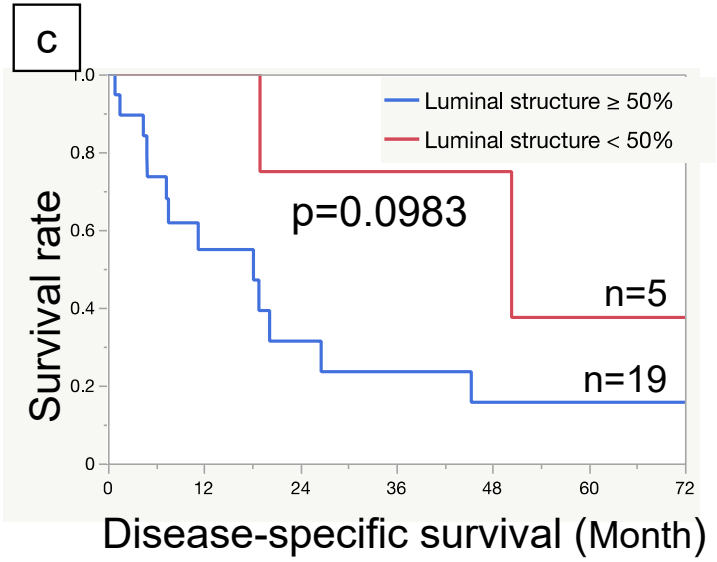
Supplementary Table 2. Antibodies used for immunohistochemistry

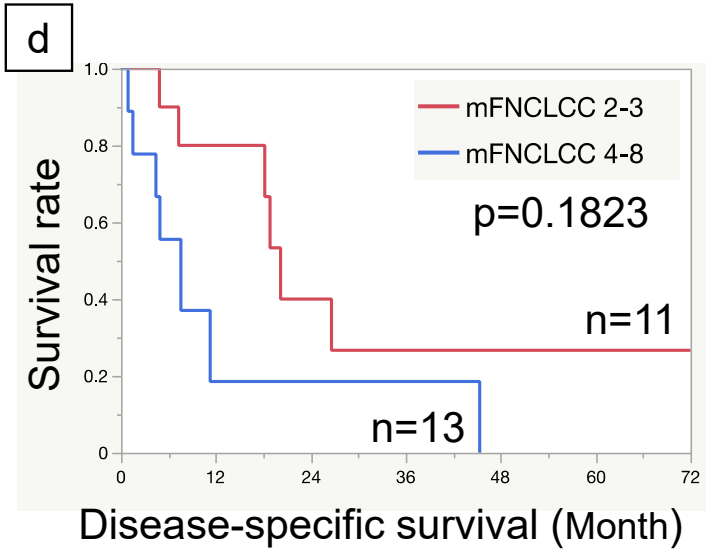
Antibody	Clone	Animal	Company	Dilution
CD31	JC70A	Mouse	Dako	1:20
CD34	QBEnd-10	Mouse	Dako	1:100
ERG	9FY	Mouse	Biocare	1:100
D2-40	D2-40	Mouse	Nichirei	Diluted
MIB-1	MIB-1	Mouse	Dako	1:100
C-MYC	Y69	Rabbit	Abcam	1:100
p16	E6H4	Mouse	Roche	Diluted
p53	DO-7	Mouse	Gene Tex	1:100
HHV-8	13B10	Mouse	Leica	1:100

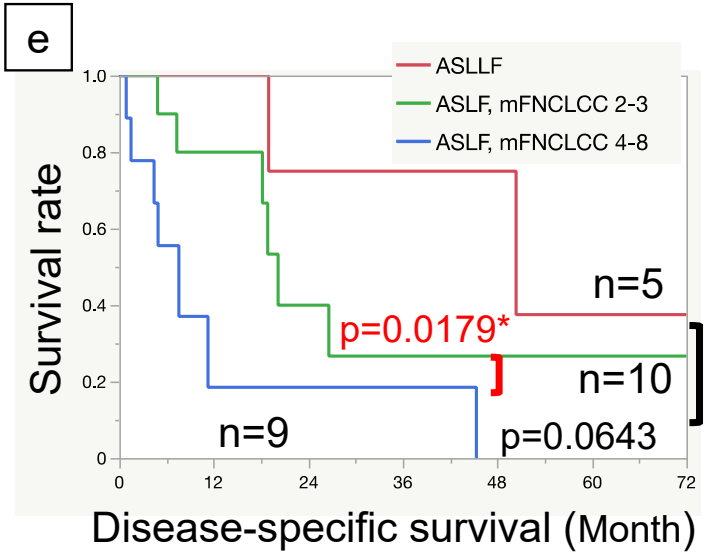
Kaplan-Meier curve (resected cases only)











Supplementary Table 4. Multivariate proportional hazard analysis on DSS

Factor	DSS		
	Hazard ratio	95% CI	*P*
Age (> 70 vs ≤ 70)	5.40	1.96-14.8	0.0011**
Primary site (Skin vs others)	0.314	0.113-0.873	0.0265*
Metastasis (Present vs absent)	9.01	3.02-26.8	<0.0001**
Size (> 5cm vs ≤ 5cm)	2.76	1.24-6.12	0.0127*
Taxane chemotherapy (Performed vs not)	0.401	0.169-0.954	0.0388*
Luminal structure (≥ 50% vs < 50%)	6.08	2.09-17.7	0.0009**
Nuclear atypia (high vs low to midium)	3.23	1.35-7.74	0.0087*

DSS indicates disease-specific survival.

* indicates P<0.05, ** indicates $P < 0.005$

Supplementary Table 5.
Relationship between luminal structure and other clinical and histological characteristics

		Luminal structure			Luminal structure			Luminal structure		
		≥30%	<30%	p	≥50%	<50%	p	≥80%	<80%	p
Age (year)	≥70	47	6		40	13		38	15	
	<70	38	12	0.1205	38	12	1.0000	35	15	1.0000
Sex	Male	54	10		49	15		45	19	
	Female	31	8	0.5965	29	10	0.8164	28	11	1.0000
Primary site	Skin	55	12		50	17		47	20	
	Other organs	31	6	1.0000	29	8	0.8116	27	10	0.8241
Mitosis (/10HPF)	>10	16	9		12	13		8	17	
	≤10	70	9	0.0120*	67	12	0.0008**	66	13	<0.0001**
Necrosis	Present	17	3		16	4		15	5	
	Absent	69	15	1.0000	63	21	0.7757	59	25	0.7880
Karyorrhexis	Present	48	15		42	21		38	25	
	Absent	38	3	0.0351*	37	4	0.0089**	36	5	0.0036**
Nuclear atypia score	3	24	13		18	19		17	20	
	1 or 2	62	5	0.0007**	61	6	<0.0001**	57	10	<0.0001**
Nucleoli score	3	23	11		18	16		17	17	
	1 or 2	63	7	0.0108*	61	9	0.0004**	57	13	0.0023**
CD31	Positive	79	13		73	19		68	24	
	Negative	3	2	0.1695	2	3	0.0752	2	3	0.1299
CD34	Positive	61	9		60	10		58	12	
	Negative	21	6	0.3465	15	12	0.0027**	12	15	0.0002**
D2-40	Positive	56	9		50	15		46	19	
	Negative	22	5	0.54	21	6	1.0000	20	7	0.8048
MIB-1 (%)	>25	30	6		27	9		22	14	
	≤25	49	7	0.7602	45	11	0.6087	45	11	0.0558
c-MYC	Positive	17	2		15	4		13	6	
	Negative	58	11	0.7256	53	16	1.00	50	19	0.7771
p53	Positive	34	7		30	11		27	14	
	Negative	43	6	0.5593	40	9	0.4461	38	11	0.2446
p16	Positive	8	4		6	6		6	56	
	Negative	63	7	0.0506	59	11	0.0145*	56	14	0.0618

HPF indicates high power field.
* indicates $P < 0.05$.
** indicates $P < 0.005$.

Kaplan-Meier curve (Angiosarcoma with less luminal formation only)

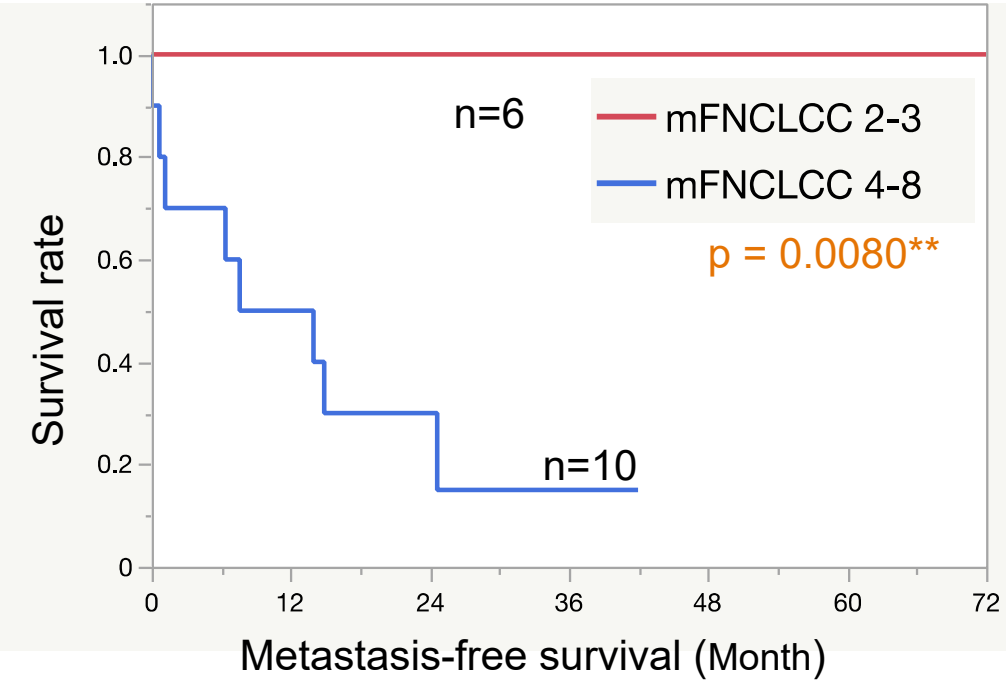


Table 1. Clinicopathological characteristics

Clinical information		Number	%	OS (p)	DSS (p)	MFS (p)
Sex	Male	65	62.5	0.0793	0.0635	0.0256*
	Female	39	37.5			
Age	Mean 68.9 years [Range 7～92 years, SD 15.0]					
	≥ 70 years old	51	49.0	0.0145*	0.004**	0.0829
	< 70 years old	53	51.0			
Primary site	Skin	67	64.4	0.0198* (Skin vs other organs)	0.0262* (Skin vs other organs)	<0.0001** (Skin vs other organs)
	Soft tissue	13	12.5			
	Mediastinum	8	7.7			
	Liver and	12	11.5			
	Others	4	3.8			
Size	< 5cm	25	40.3	0.0664	0.0628	0.9053
	≥ 5cm	37	59.7			
Metastasis	Present	15	23.4	<0.0001**	<0.0001**	NA
	Absent	49	76.6			
Resection	Performed	15	25.4	0.1471	0.1992	0.8899
	Not performed	44	74.6			
Radiation	Performed	41	69.5	0.9159	0.8191	0.7823
	Not performed	18	30.5			
Taxane chemotherapy	Performed	28	47.5	0.0030**	0.0021**	0.0445*
	Not performed	31	52.5			
Reason of death	Tumor death	42	61.8	NA	NA	NA
	Other reason	26	38.2			

OS indicates overall survival; DSS, disease-specific survival; MFS, metastasis-free survival; SD, standard deviation; NA, not applicable.
* indicates p <0.05.
** indicates p <0.005.

Table 2. Histological and immunohistochemical results

				OS		DSS		MFS	
		Number	%	Number of available cases	p	Number of available cases	p	Number of available cases	p
Nuclear atypia score	1	27	26.0		0.5246		0.4493		0.0090**
	2	40	38.5	48	(1-2 vs 3)	48	(1-2 vs 3)	38	(1-2 vs 3)
	3	37	35.6	20		20		19	
Nucleoli score	1	36	34.6		0.1459		0.0901		0.9050
	2	34	32.7	50	(1-2 vs 3)	50	(1-2 vs 3)	41	(1-2 vs 3)
	3	34	32.7	18		18		16	
Luminal structure	≥30%	86	82.7	47	0.0236*	57	0.0399*	46	0.0826
	<30%	18	17.3	11		11		11	
	≥50%	79	76.0	52	0.0240*	52	0.0330*	41	0.0893
	<50%	25	24.0	16		16		14	
	≥80%	74	71.2	48	0.0553	48	0.0678	38	0.1607
	<80%	30	28.8	20		20		19	
Conventional solid pattern	Present	20	19.2	12	0.0724	12	0.077	12	0.2235
	Absent	84	80.8	56		56		45	
Epithelioid feature	Present	25	24.0	12	0.5041	12	0.6135	12	0.2226
	Absent	79	76.0	56		56		45	
Mitosis score	1	79	76.0	54	0.8850	54	0.9802	44	0.0149*
	2	15	14.4						
	3	10	9.6	14	(1 vs 2-3)	14	(1 vs 2-3)	13	(1 vs 2-3)
Necrosis score	0	84	80.8	59	0.0267*	59	0.0175*	41	0.0169*
	1	17	16.3						
	2	3	2.9	9	(0 vs 1-2)	9	(0 vs 1-2)	6	(0 vs 1-2)
Karyorrhexis	Present	63	60.6	39	0.8268	39	0.6574	40	0.6574
	Absent	41	39.4	29		29		29	
CD31	Positive	91	95.8	61	0.8821	61	0.7626	50	0.9144
	Negative	4	4.2	3		3		3	
CD34	Positive	70	72.2	45	0.5012	45	0.6087	38	0.4296
	Negative	27	27.8	19		19		15	
ERG	Positive	95	100.0	64	NA	64	NA	64	NA
	Negative	0	0.0	0		0		0	
D2-40	Positive	65	70.7	49	0.3196	49	0.1669	42	0.1077
	Negative	27	29.3	12		12		8	
MIB-1 (%)	≥ 25	36	39.1	26	0.4708	26	0.3155	22	0.2603
	< 25	56	60.9	36		36		29	
c-MYC	Positive	19	21.6	11	0.0479*	11	0.1903	9	0.0084**
	Negative	69	78.4	48		48		41	
p16	Positive	17	18.9	15	0.0367*	15	0.0636	14	0.0312*
	Negative	73	81.1	46		46		37	
p53	Positive	50	61.0	36	0.7905	36	0.9054	33	0.0598
	Negative	32	39.0	21		21		15	

OS indicates overall survival; DSS, disease-specific survival; MFS, metastasis-free survival; NA, not applicable.
* indicates p <0.05.
** indicates p <0.005.