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CD8$^+$tumor-infiltrating lymphocytes at primary sites as a possible prognostic factor of cutaneous angiosarcoma

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Tumor-infiltrating lymphocytes (TILs) have been reported as a prognostic factor in various cancers and are a promising target for immunotherapy. To investigate whether TILs have any impact on the prognosis of angiosarcoma patients, 55 non-treated patients (40 patients at stage 1 with cutaneous localized tumors, 4 patients at stage 2 with lymph node metastases and 11 patients at stage 3 with distant metastases) with angiosarcoma were evaluated retrospectively by immunohistochemistry stained CD4, CD8, FOXP3 and Ki67. The Kaplan–Meier method was used to estimate overall survival with patients at stage 1. Survival differences were analyzed by the log-rank test. Patients with higher numbers of CD8$^+$ TILs in their primary tumors survived significantly longer compared with patients with lower values. Moreover, the number of CD8 in TILs was positively correlated with a distant metastasis-free period. The total number of primary TILs (CD4 plus CD8) and CD8$^+$ primary TILs of stage 3 patients with distant metastases was positively correlated with their overall survival. To evaluate whether CD8$^+$ effector T cells are activated or differentiated, flow cytometric analysis of peripheral blood mononuclear cells (PBMC) was performed. The percentages of CD8$^+$ T cells producing IFN-γ in PBMC were significantly higher in patients with angiosarcoma ($n = 10$) compared not only with that of healthy controls ($n = 20$) but also patients with advanced melanoma ($n = 11$). These results suggest that anti-tumor immunity is clinically relevant in angiosarcoma.

Cutaneous angiosarcoma is a rare soft-tissue sarcoma of endothelial cell origin that has a poor prognosis.[1] Although angiosarcoma can arise anywhere in the body, it often arises on the face and scalp, leading to a poor prognosis and decreased quality of life. Chemotherapies and radiotherapies are options for angiosarcoma treatment; however, unfortunately, there is no treatment that cures angiosarcoma except complete resection, which is often not possible. A more effective therapy is required to improve the prognosis of angiosarcoma, and immunotherapy may be one of the candidate therapies. Indeed, some previous studies suggest the importance of tumor immunity in the progression of angiosarcoma. Maddox and Evans reported that in patients with angiosarcoma, those with moderate or marked inflammation in tumors showed significantly better survival compared with those with slight or no inflammation.[2] Furue *et al.* reported a case of angiosarcoma in which immunotherapy using tumor-infiltrating lymphocytes (TILs) for Stewart–Treves syndrome dramatically improved the clinical symptoms.[3] Zietz *et al.* reported that angiosarcoma with low Fas-L expression was characterized by numerous TILs and the survival durations of patients with high Fas-L expressing angiosarcomas were significantly reduced compared to patients with low Fas-L-expressing tumors.[4] These reports suggest the importance of TILs in the prognosis of angiosarcoma patients,

Key words: cutaneous angiosarcoma, tumor-infiltrating lymphocytes, prognosis

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What's new?

Tumor-infiltrating lymphocytes (TILs) have been reported to be a prognostic factor in various cancers. In this study, the authors investigated whether TILs have any impact on the prognosis of angiosarcoma. They found that higher numbers of $CD8^+$ TILs correlated with both a longer period free of distant metastases, and a more favourable prognosis. PBMC analysis showed that patients with angiosarcoma also had higher numbers of $CD8^+$ effector T cells than did healthy controls. TIL-based immunotherapy may thus provide a promising approach to the treatment of angiosarcoma.

and that the evaluation of tumor immunity may be possible by analyzing TILs.

Recent clinical experience in immunotherapy indicates that anti-tumor immunity mediated by T cells can have a strong impact on the clinical course of certain tumors.[5–7] Effective cancer immunotherapy is dependent on the presence of large numbers of anti-tumor lymphocytes with appropriate homing and effector functions that enable them to seek out and destroy cancer cells *in vivo*.[5] TILs have been reported as one of the representative manifestations of host anti-tumor immune response and as a target for immunotherapy.[5,8,9] Indeed, TILs are associated with the prognosis of various cancers.[10–15] In addition, the analysis of T cell subsets in TILs can provide a deeper insight into anti-tumor immunity. In particular, $CD8^+$ effector T cells in TILs have associations with the prognosis of various cancers.[12,14,16–20] Conversely, the ratio of $FOXP3^+$ regulatory T cells (Tregs) in TILs is reported to have correlations with a worse prognosis in patients with certain cancers.[21,22]

Although tumor immunity can have an impact on the prognosis of angiosarcoma, the lymphocyte subsets of TILs and peripheral blood mononuclear cells (PBMC) in angiosarcoma patients have not been fully characterized. In this study, by means of immunohistochemistry, we have investigated whether the subsets of TILs have correlations with the clinical course or prognosis of patients with angiosarcoma. Moreover, to compare the frequencies of effector T cells in peripheral blood with healthy controls and other malignant cutaneous tumors, the percentage and IFN-γ production of $CD8^+$, $CD4^+$ T cells in PBMC of angiosarcoma, melanoma and healthy controls were assessed by flow cytometric analysis.

Material and Methods

Patient samples

Blood and tissue samples were obtained from Japanese patients with angiosarcoma (fresh blood samples $n = 10$, paraffin-embedded tissue samples $n = 55$), healthy controls (fresh blood samples age-sex matched $n = 20$) and melanoma patients (fresh blood samples, $n = 11$). We retrospectively checked paraffin-embedded tissue samples (resected as biopsies or surgeries) and also medical records from Kyoto University Hospital, and found 29 patients with angiosarcoma from 1990 to 2012. In this article, we used tissues of non-treated primary cutaneous tumors. Thus, we excluded four samples, which had already received some treatment. We obtained 25 samples from the University of the Ryukyu, 2 samples from Fukui Red Cross Hospital and 3 samples from Kyoto City Hospital. Thus, we arrived at a total of 55 samples.

This study was approved by the Medical Ethics Committee of the Kyoto University Graduate School of Medicine, and was conducted in accordance with the principles of the Declaration of Helsinki. All patients and healthy controls provided written informed consent to participate in the study.

Patient's characteristics: clinical stages and the number of TILs

There had been no certain clinical tumor staging strategy for cutaneous angiosarcoma that reflected the prognosis or curative effect throughout the world. In this study, we classified our patients groups simply into three stages by modifying classic TNM classification as reported previously, stage 1 for those with cutaneous local tumors, 2 for those with lymph node metastases and 3 for those with distant metastases,[23,24] which reflected the patients' prognosis. Our 55 non-treated patients (40 stage 1, 4 stage 2 and 11 stage 3 patients) with angiosarcoma were consistent with the previous report[23,24] with the overall survival being different in each stage (Stage1-$> 2 >$ 3) as estimated by the Kaplan–Meier method (data not shown for stage 2).

Non-treated primary tumor specimens of 55 patients were evaluated retrospectively by immunohistochemistry stained CD4, CD8, FOXP3 ($n = 55$), MHC class1 ($n = 52$), Ki67 ($n = 45$) and CD31 ($n = 51$). We performed Ki67 staining as a proliferation marker, calculating the Ki67-index of tumors, and using CD31 staining as a vascular endothelial cell marker to aid in the detection of tumors. Count data and patients' characteristics are presented in Table 1.

Flow cytometric analysis

Flow cytometric analyses for PBMC of patients with untreated stage 1 angiosarcoma were planned and performed from 2007 to 2012 in Kyoto University Hospital. The total sample number was 10. PBMC were isolated with Ficoll-Isopaque (Lymphoprep™; Axis-Shield, Oslo, Norway) gradient centrifugation. PBMC were freshly stained and analyzed with the following monoclonal antibodies as previously described[25]: allophycocyanin (APC) conjugated anti-CD4 (clone 11830; R and D systems); fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ (clone 4S.B3; BD Biosciences); peridinin chlorophyll protein-Cy5.5 (PerCp-Cy5.5)-conjugated anti-CD8 (clone SK1; BD Biosciences).

Table 1. Patients characteristics

Pt.	Survival month	Stage	Age	Sex	Anatomic distribution	distant metastasis-free months (mainly Lung)	Metastasis	Therapy	CD4	CD8	CD4+CD8	FOXP3	Ki67-index(%)
1	13#	I	93	F	Scalp			S,RT,rIL-2	49.0	88.3	137.3	14.0	12.3
2	21#	I	74	M	face	16	Lung, Liver, Bone	RT	257.2	321.6	578.8	55.4	38.0
3	39	I	63	M	face			S,RT	94.6	127.6	222.2	27.8	14.0
4	7#	I	93	F	Scalp			rIL-2,RT	158.3	111.5	269.8	12.5	10.1
5	13#	I	78	F	Scalp			RT,CT	72.0	67.6	139.6	5.6	*
6	15#	I	86	M	Scalp			rIL-2,RT	122.0	297.0	419.0	85.0	34.3
7	15#	I	79	F	Scalp	2	Lung, Lymphnode	S,RT,CT,rIL-2	129.0	137.5	266.5	30.8	9.4
8	16#	I	76	M	Scalp	5		rIL-2,RT,CT	43.6	48.6	92.2	3.6	2.8
9	17#	I	78	M	Scalp			RT,CT	65.8	78.3	144.0	16.3	*
10	19	I	85	M	Scalp	8	Lung, parotid gland	S,RT	323.4	221.4	544.8	15.6	3.8
11	20#	I	87	F	Scalp			rIL-2,RT	62.5	131.8	194.3	13.0	*
12	24#	I	71	M	Scalp	23	Lung, Liver, Bone	rIL-2,RT	148.5	228.0	376.5	75.8	9.7
13	70	I	75	F	Scalp			S,RT,CT,rIL-2	216.0	346.0	562.0	59.3	12.4
14	84#	I	86	M	Scalp,face	84	Lung	S,RT,rIL-2	29.0	76.5	105.5	12.7	8.0
15	5	I	81	M	Scalp			RT	47.0	56.0	103.0	16.0	16.6
16	40 (D)	I	56	F	lower extremity			rIL-2	107.8	196.0	303.8	15.0	7.1
17	6#	I	73	M	Scalp	2	Lung	rIL-2	61.0	58.0	119.0	20.0	4.8
18	6#	I	75	F	Scalp	5	Lung	rIL-2,S,RT	12.5	26.3	38.8	5.3	2.6
19	13#	I	58	M	Scalp	11	Lung, Lymphnode	rIL-2,S,CT,RT	87.4	97.8	185.2	24.4	17.2
20	5#	I	71	M	Scalp	2	Lung	rIL-2,S,RT	148.0	67.0	215.0	21.8	13.1
21	38#	I	64	M	forehead	34	Parotid gland, Lung	rIL-2,RT	156.8	252.0	408.8	49.2	*
22	100#	I	70	F	Scalp			rIL-2,S,CT,RT	56.6	41.8	98.4	8.8	13
23	89	I	50	M	Scalp			rIL-2,S	54.7	94.0	148.7	33.7	13.8
24	79#	I	77	F	chest	70	Lung, Bone	rIL-2,S	203.7	147.3	351.0	43.7	*
25	10#	I	52	F	lower abdomen	6	Lung	rIL-2,CT,RT	101.6	58.0	159.6	26.8	56.3
26	19#	I	58	M	Scalp	10	Lung	rIL-2,S,CT,RT	232.0	91.0	323.0	32.6	*
27	7#	I	77	M	Scalp	6	Lung	rIL-2,S,CT,RT	109.3	34.3	143.5	36.0	21.5
28	7 (R)	I	63	M	Scalp			P	73.2	63.2	136.4	9.0	37.9
29	15#	I	73	F	Scalp	9	Lung,Brain	rIL-2,P	398.7	85.7	484.3	56.0	22.5
30	31#	I	70	M	Scalp	21	Lung	rIL-2,CT,RT	165.2	102.6	267.8	32.6	10.2
31	14#	I	84	M	Scalp	12	Lung	rIL-2,CT,RT	125.8	66.4	192.2	17.0	27.8
32	19	I	82	F	Scalp			CT	349.0	108.4	457.4	66.8	36.7

Table 1. Patients characteristics (Continued)

Pt.	Survival month	Stage	Age	Sex	Anatomic distribution	distant metastasis-free months (mainly Lung)	Metastasis	Therapy	CD4	CD8	CD4+CD8	FOXP3	Ki67-index(%)
33	32#	I	78	M	Scalp	26	Liver,Spleen	rIL-2,S,CT,RT	149.0	108.8	257.8	27.8	18.3
34	21	I	62	M	Scalp			S,CT	201.2	155.2	356.4	36.0	*
35	6#	I	88	M	Scalp			S,CT	143.4	73.2	216.6	30.4	20.7
36	27	I	52	M	forehead			rIL-2,S	157.0	79.4	236.4	28.8	*
37	7	I	81	M	Scalp			S,CT	257.6	253.2	510.8	16.2	*
38	7	I	64	F	chest			S,CT	215.6	85.8	301.4	17.6	*
39	7	I	59	M	Scalp			S,CT	249	80.6	329.6	42.6	41.9
40	4	I	86	M	Scalp			S,CT	114.6	258.8	373.4	43.6	21.8
41	3#	II	75	M	Scalp	-	Lymph node	CT,RT,rIL-2	144.2	162.4	306.6	14.6	39.1
42	11	II	63	F	Scalp	-	Lymph node	S,CT	373.6	153.2	526.8	31.8	36.7
43	43#	II	58	F	Scalp	-	Lymph node	RT,CT	374.3	109.6	483.9	44.8	39.2
44	26	II	62	F	Chest	-	Lymph node	S,CT	286	189	475	107.6	31.7
45	2#	III	87	F	Scalp	-	Lung	S,rIL-2,CT	276.6	100.0	330.8	45.0	16.1
46	4#	III	70	M	Scalp,face	-	Lung	RT,CT	68.0	80.7	148.7	21.0	9.0
47	5#	III	75	M	Scalp	-	Lung, Liver,Spleen	S,CT,RT	77.8	225.3	303.0	26.5	21.4
48	6#	III	78	M	Scalp	-	Lung	S,RT,rIL-2	38.0	117.5	155.5	16.5	4.2
49	11#	III	79	M	Scalp	-	Lung	rIL-2	80.6	92.6	173.2	7.8	25.1
50	12#	III	75	M	Scalp	-	Lung, Liver,Spleen	rIL-2	163.0	208.0	371.0	9.0	6.5
51	15#	III	93	F	face	-	Lung	S,RT	197.8	252.0	449.8	75.8	24.0
52	16#	III	75	M	Scalp	-	Lung	CT,RT,rIL-2	277.8	178.3	456.0	22.8	1.8
53	29#	III	58	M	Scalp	-	Lung	RT,CT,rIL-2	225.5	251.8	477.3	61.5	39.8
54	2#	III	74	M	Scalp	-	Lung, liver, spleen,small intestine.	rIL-2	78.2	50.4	128.6	16.8	7.6
55	9#	III	67	M	Scalp	-	Lung, Bone	CT	89	58	148	10.2	12.1

Abbreviations: M, male; F, female; D, drop out of the follow up at 40 months, R, tumor was completely regressed but died for another reason. Pt.:patient number
means "already dead from angiosarcoma".
TILs, tumor infiltrating lymphocyte = CD4/HPF + CD8/HPF; % FOXP3, FOXP3/CD4 × 100; rIL-2, recombinant interleukin-2; S, surgery; CT, chemotherapy; RT, radiation therapy; P, phenol application
Excluded samples of Ki67-index (%)*: 21,26,36: not stained because of no sample. 5,9,11,24,34,37,38: difficult to count Ki67-index because of poor staining or not enough tumor cells to keep quality of count ki67-index.

Acquisition and data analysis were performed with FACS Calibur (BD Biosciences) or LSR Fortessa (BD Biosciences) and Flow Jo software (Tree Star).

Intracellular cytokine staining

Fresh PBMC (5×10^6 cells/well) in 24-well plates were stimulated with 20 ng/ml PMA and 1 μM ionomycin in the presence of protein transport inhibitor monensin (Golgi-Stop, BD Biosciences) for 5 hr. Cells were harvested and co-stained with anti-CD4 and anti-CD8 monoclonal antibodies for surface staining. Then, cells were fixed, permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with anti-IFN-γ monoclonal antibodies for intracellular staining prior to analysis by flow cytometry.

Immunohistochemistry

Tumor-infiltrating lymphocytes (TILs) were characterized in 3 μm-thick serial sections cut from formalin-fixed, paraffin-embedded, tissue specimens of the most representative tumor areas. Immunohistochemistry by autoclave antigen retrieval methods was carried out as previously described.[26] The following primary anti-human monoclonal antibodies were used: anti-CD4 (clone 1F6; Novocastra, UK), anti-CD8 (clone C8/144B; Dako, Denmark), anti-FOXP3 (clone 236A/E7; eBioscience), anti-Ki67 (clone MM1; Novocastra, UK), anti-CD31 (clone 1A10; Novocastra, UK) and anti-MHC class1 (anti-HLA class I [HLA-A, B, C], clone EMR8-5, MBL Tokyo Japan).

Quantification of the tumor-infiltrating lymphocytes

In each immunostained serial section, the entire tumor area was evaluated for TILs at a scanning power (25× objective and 10× eyepiece). Areas with the most abundant TILs were selected, and a maximum of five randomly chosen high power field (HPF) (40× objective and 10× eyepiece) were digitally photographed and counted manually. The count was performed two times for each photograph by the same investigator (H.F.) without knowledge of earlier results. In all cases, absolute numbers of TILs (add $CD4^+$ T cell count and $CD8^+$ T cell count), $CD4^+$, $CD8^+$, $FOXP3^+$ tumor infiltrate lymphocytes per HPF, % FOXP3 lymphocyte ratio ($FOXP3^+$ T cell count divided by $CD4^+$ T cell count and expressed in percentage terms), CD8/CD4 lymphocyte ratio ($CD8^+$ T cell count divided by $CD4^+$ T cell count) and CD8 / FOXP3 ($CD8^+$ T cell count divided by $FOXP3^+$ T cell count) were calculated. The average TILs count for each patient was used for statistical analysis. The Ki67-index was also calculated. All the immunohistochemical Ki67 stained glass slides were reviewed by our dermatopathologist (S.S.). The Ki67-index of tumor was defined as the percentage of tumor cells staining positive for the Ki67 antigen in the total number of tumor cells, by counting multiple regions with highest labeling density. To keep the index quality, we excluded bad Ki67 staining sections, in which it was difficult to distinguish tumor cells from surrounding connected tissues, or in which more than 500 tumor cells could not be counted.

Statistical analysis

Overall survival was measured as the time from the patient's first visit to the hospital to the time of death or the time the patient was last seen (until August 2012). For all immunohistochemical markers, cutoff for definition of subgroups was the median. The log-rank test was used to perform univariate analyses and the survival curves were estimated by the Kaplan–Meier method. The Mann–Whitney *U*-test was used for comparing unpaired flow cytometric data of two groups. The Kruskal–Wallis *H*-test was used for multiple comparisons. Correlations were evaluated using the Spearman's rank correlation coefficient. p values were two tailed and a p value < 0.05 was considered statistically significant.

Results

Prognostic significance of primary TILs in patients with stage 1 cutaneous angiosarcoma

To investigate whether the numbers of TILs and T cell subsets of TILs at cutaneous primary sites have a relationship with prognosis of patients with cutaneous angiosarcoma, immunohistochemical analysis of TILs of angiosarcoma was performed.

Figure 1*a* shows the survival curve of our 40 non-treated stage 1 patients with angiosarcoma (Fig. 1*a*). Immunohistochemical analysis of their primary tumors is done for CD31, Ki67, MHC class 1, CD4, CD8 and FOXP3 (Fig. 1*b*). The patients with stage 1 cutaneous angiosarcoma were divided into two groups using the median value as the cutoff for each variable. The prognostic significance between the T cell subsets of TILs and their overall survival was calculated by the Kaplan–Meier method. Patients with higher numbers of CD8/HPF (high power field, high; $n = 20$, low; $n = 20$, $p = 0.019$; Fig. 1*c*) in their primary tumors demonstrated improved survival compared with patients with lower frequencies. No significant association was found for TILs/HPF (Fig. 1*c*), CD4/HPF (Fig. 1*c*), FOXP3/HPF (Fig. 1*c*), ratio of CD8/CD4, FOXP3/CD4 (%FOXP3), CD8/FOXP3 of T cell subsets and Ki67-index of tumors (data not shown).

Next, to examine how the $CD8^+$ TILs contributed to the better prognosis of patients with angiosarcoma, we evaluated their disease-free survival. However, it was difficult for most patients to maintain tumor-free status because angiosarcoma spreads with unclear borders, so we could not clearly evaluate the disease-free survival. Clinical histories revealed that most patients with angiosarcoma died from lung metastases. Therefore, we chose to evaluate the relation between distant metastases (mainly lung) and TILs. Indeed, the periods between their initial visits to our hospital and distant metastases (distant metastasis-free period) were closely correlated with their overall survival ($n = 18$, $p = 2.13\text{E-}06$, $r = 0.874$; data not shown). Moreover, CD8/HPF of TILs was positively correlated with the distant metastasis-free period ($n = 19$,

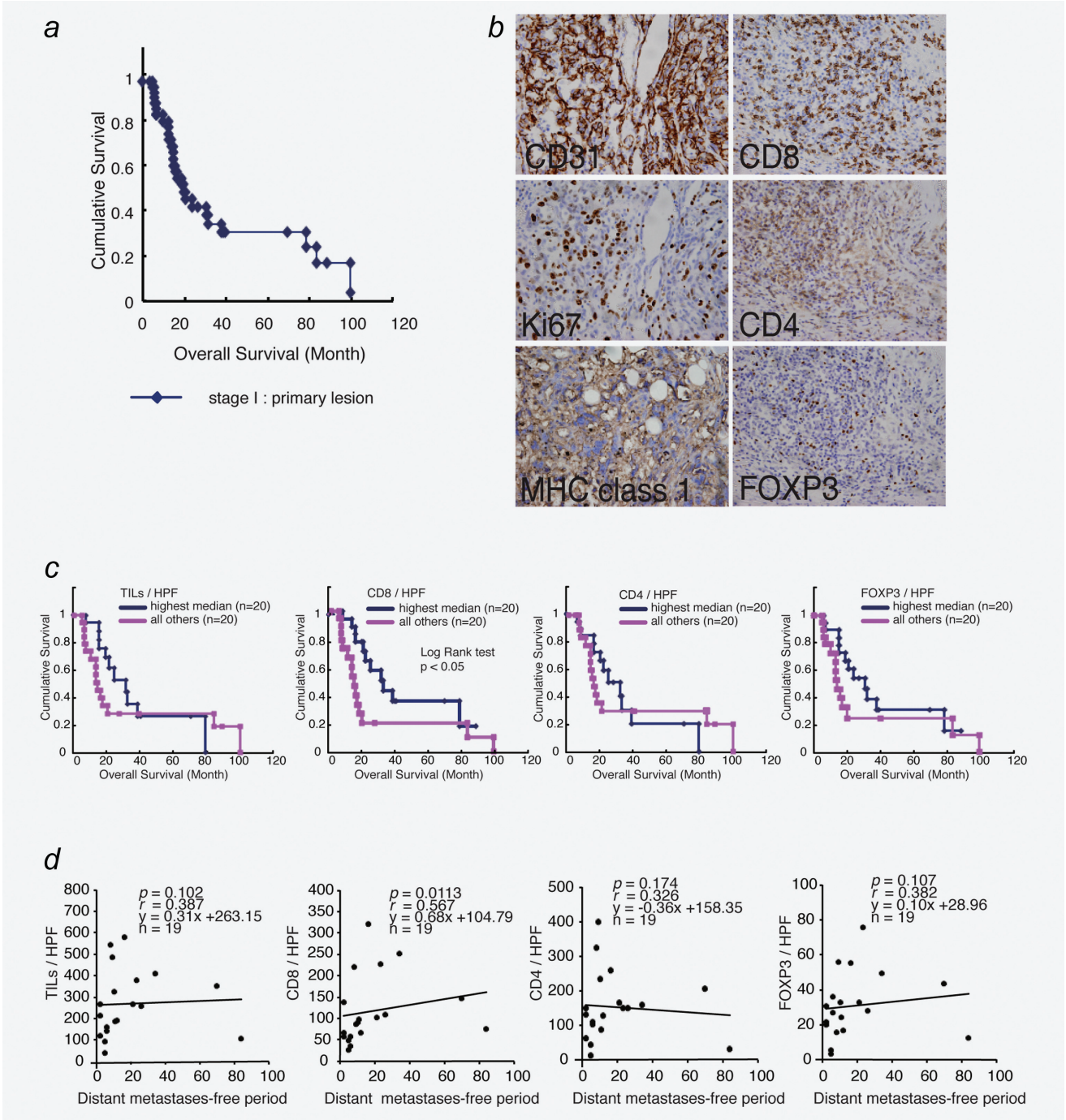

Figure 1. Prognostic significance of primary TILs in patients with stage 1 cutaneous angiosarcoma. The survival curve of our 40 non-treated stage 1 patients with angiosarcoma (*a*). Representative immunohistochemistry was presented (*b*). The stage 1 patients were divided into two groups using the median value as the cutoff of each variable. The Kaplan–Meier method was used to estimate overall survival. Survival differences were analyzed by the log-rank test based on the number of TILs (CD8/HPF plus CD4/HPF), its subsets CD8/HPF, CD4/HPF and FOXP3/HPF (*c*). Correlation analysis was performed by Spearman rank correlation test with clinical parameters described below and frequencies of TILs. Primary TILs in stage 1 patients were used for the statistic. There was positive correlation between the months from initial visit to the hospital until patients developed distant metastases (in the graph, name as distant metastasis-free period) and the overall survival period of those patients who were deceased ($n = 18$, data not shown). Correlation between distant metastasis-free period and the number of TILs (CD8/HPF plus CD4/HPF), its subsets CD8/HPF, CD4/HPF and FOXP3/HPF (*c*).

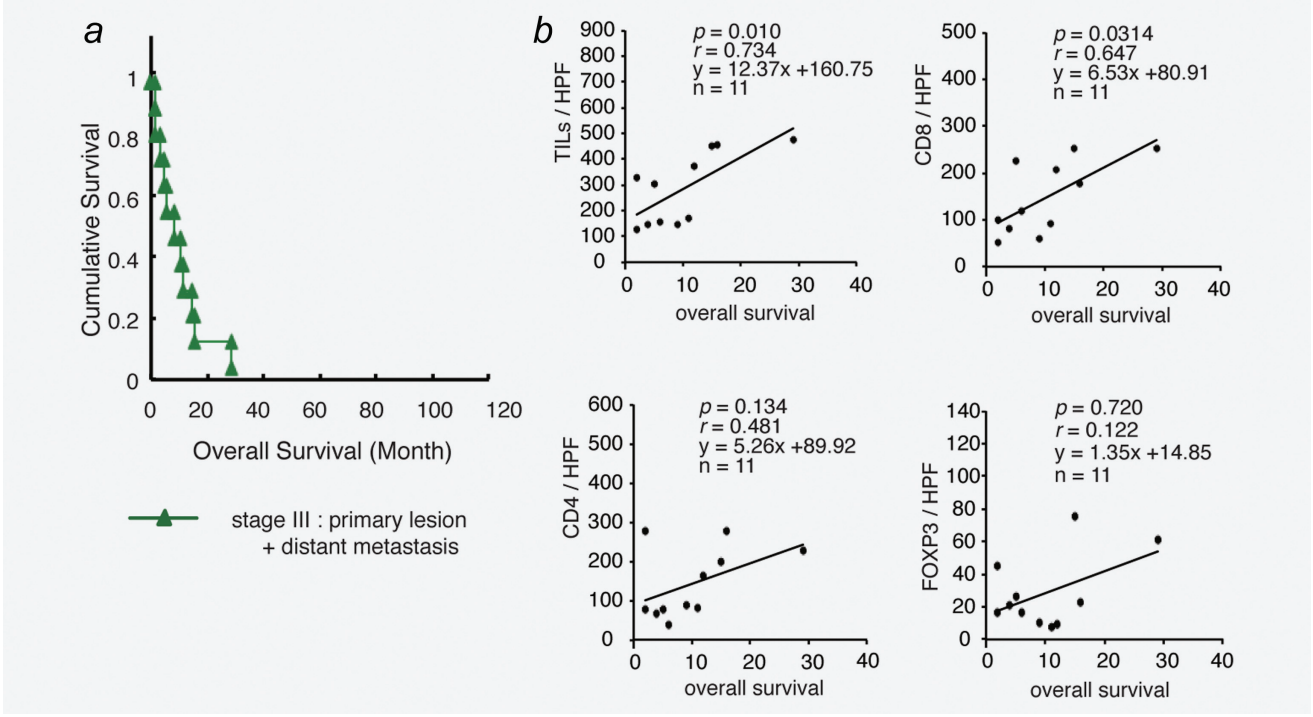

Figure 2. Significant correlation between primary TILs in stage 3 patients and overall survival. The survival curve of our 11 non-treated stage 3 patients with angiosarcoma (*a*). Correlation analysis was performed by spearman rank correlation test with overall survival and frequencies of TILs in deceased patients ($n = 11$). TILs (CD8/HPF plus CD4/HPF), CD8/HPF, CD4/HPF and FOXP3/HPF (*b*).

$p = 0.0113$, $r = 0.567$; Fig. 1*d*). No other immunohistological parameters had significant correlations with the distant metastasis-free period (Fig. 1*d*, and data not shown).

Significant correlation between primary TILs in stage 3 patients and overall survival

Next, we evaluated correlation analysis with the prognosis of stage 3 patients who developed distant metastases, although we could not assess stage 2 patients who developed lymph node metastases because of the small number of patients.

Figure 2*a* shows the survival curve of our 11 non-treated stage 3 patients with angiosarcoma (Fig. 2*a*).

All 11 patients with stage 3 angiosarcoma had already died from the tumors. The number of primary TILs (CD4 plus CD8) and $CD8^+$ primary TILs of stage 3 patients was positively correlated with their overall survival (TILs; $n = 11$, $p = 0.010$, $r = 0.734$, Fig. 2*b*, CD8; $n = 11$, $p = 0.0314$, $r = 0.647$, Fig. 2*b*).

The percentage of IFN-γ production of $CD8^+$ T cells in PBMC of patients with angiosarcoma

To evaluate whether T cells, especially CD8 effector T cells, are activated or differentiated in patients with angiosarcoma, we analyzed IFN-γ production of $CD4^+$ and $CD8^+$ T cells in peripheral blood mononuclear cells (PBMC) using flow cytometry in available non-treated patients with cutaneous angiosarcoma ($n = 10$, stage1) and compared it with healthy controls (HC, $n = 20$) and patients with malignant melanoma (MM, $n = 11$, stages 3 and 4) as a disease control.

The percentages of $CD8^+$ T cells producing IFN-γ in PBMC were significantly higher in patients with angiosarcoma compared with those of healthy controls and patients with melanoma (IFN-γ; median, HC 56.0% *vs.* AS 76.8% *vs.* MM 64.3%, $p = 0.0295$ compared three groups by Kruskal–Wallis *H*-test, AS > HC, $p = 0.0326$, AS > MM $p = 0.0296$ by Mann–Whitney *U* test. Fig. 3*b*). Moreover, the percentage of IFN-γ producing $CD4^+$ T cells was also significantly higher in angiosarcoma patients compared with those of healthy controls, but was not significant compared with those of melanoma patients (IFN-γ; median, HC 12.8% *vs.* AS 23.7% *vs.* MM 16.5%, $p = 0.00992$ compared three groups by Kruskal–Wallis *H*-test, AS > HC, $p = 0.00324$, AS > MM $p = 0.0884$ by Mann–Whitney *U* test, Fig. 3*b*).

These results indicated that PBMC in angiosarcoma patients contained significantly larger frequencies of $CD8^+$ and $CD4^+$ effector T cells.

Discussion

To the best of our knowledge, this study provides the first immunological data showing that primary $CD8^+$ TILs are the most relevant T cell population in the prognosis of cutaneous angiosarcoma. Previous reports[2,4] showed that higher numbers of TILs correlated with a favorable prognosis in patients with angiosarcoma. Our study examined

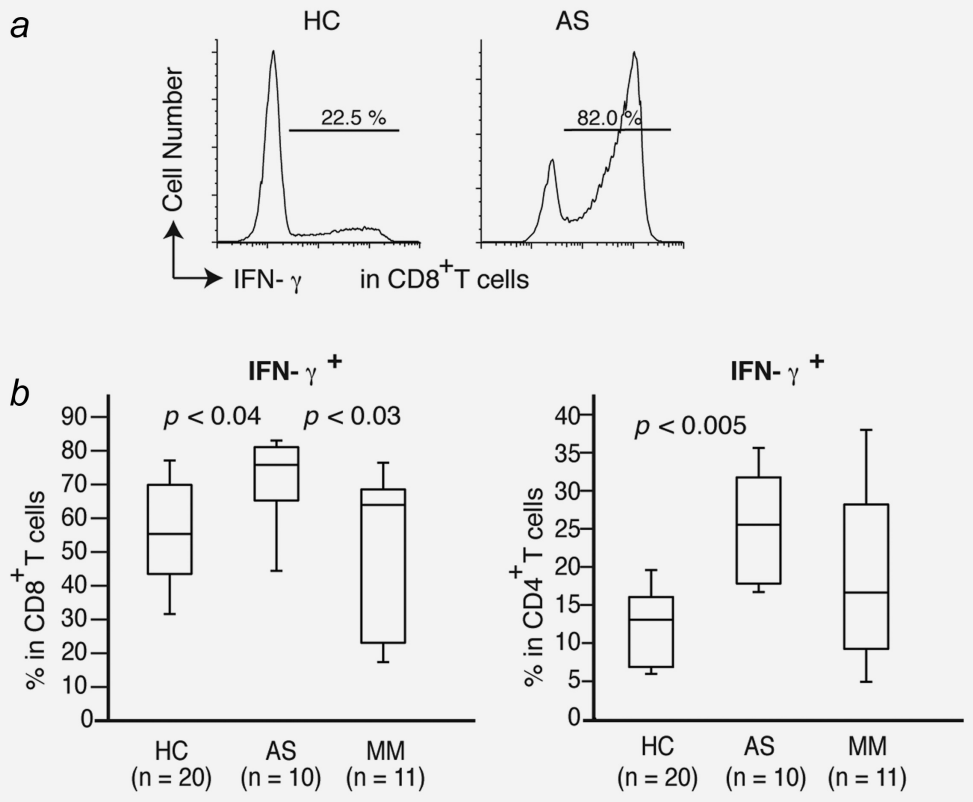

Figure 3. The percentage of IFN-γ production of $CD8^+$ T cell in PBMC of patients with angiosarcoma. Freshly isolated PBMC from patients with angiosarcoma (AS; $n = 10$, stage 1), healthy controls (HC; n = 20, age-matched) and patient with malignant melanoma (MM; $n = 11$, stages 3 and 4) were stimulated with PMA and ionomycin combined with a protein transport inhibitor for 5 hr. Cells were co-stained with anti-CD4, -CD8 and anti-IFN-γ antibodies, and analyzed by flow cytometry. Representative flow cytometric histograms of IFN-γ^+ cells in $CD8^+$ T cells (*a*). Percentages of cytokine-positive cells were shown. The percentages of IFN-γ^+ cells in $CD8^+$ T cells (AS; $n = 10$, HC; $n = 20$, MM; $n = 11$), or in $CD4^+$ T cells (AS; $n = 10$, HC; $n = 20$, MM; $n = 11$) were shown (*b*). The Kruskal–Wallis *H*-test was used for multiple comparisons. The Mann–Whitney *U*-test was used for comparing unpaired data.

immunological parameters of TILs including CD4, CD8, FOXP3 and found that CD8 is the most possible prognostic factor.

Galon *et al.* proposed a new cancer classification using "immunoscore," which is a tool including immunological biomarkers to predict prognosis and response to therapy.[27] The study stated that immunoscore should be incorporated into the traditional TNM classification. Our results indicated that tumor-infiltrated CD8 can be one parameter of the immunoscore in patients with angiosarcoma.

In our data, higher numbers of $CD8^+$ TILs resulted in a longer distant metastasis-free period. This result suggested that $CD8^+$ cytotoxic T lymphocytes in TILs are important in suppressing progression of angiosarcoma. It has been demonstrated in animal models that infiltration of tumors by tumor-reactive T lymphocytes is required for efficient tumor regression[28,29] and the concentration of $CD8^+$ T cells determined the efficiency of target cell killing.[30] In human studies, accumulating evidence has shown that tumor infiltration by $CD8^+$ T cells is associated with a better prognosis in various cancers, such as bladder cancer,[12] renal cell carcinoma,[16] colorectal cancer,[14] ovarian cancer,[17,18] esophageal carcinoma[19] and hepatocellular carcinoma.[20] $CD8^+$ cytotoxic T lymphocytes recognizing tumor antigens are also found in human melanoma-specific TILs,[31] and $CD8^+$ TILs cultured from metastatic melanoma exerted lytic activity on autologous melanoma cells.[31,32] Therefore, tumor infiltrating $CD8^+$T lymphocytes have been recognized to have antitumor effects on cancer patients. Together with our data, the contribution of $CD8^+$ TILs to the favorable prognosis in angiosarcoma seemed mostly due to the ability of $CD8^+$ T cells as tumor antigen-specific cytotoxic T lymphocytes to kill target cells directly, although the *in vitro* ionomycin assay evaluated in this study is only by PBMC and is not a conclusive experiment to prove the speculation.

To recognize a tumor, $CD8^+$ T cells need the expression of MHC class 1 on tumor cells. It was previously reported, through the analysis of a few cases, that angiosarcoma expressed both MHC class 1 and cancer-testis-antigen.[33] In this report, all the analyzed samples of angiosarcoma expressed MHC class 1 ($n = 52$ data not shown). After recognition of a tumor, one of the mechanisms of tumor lysis by $CD8^+$ cytotoxic T lymphocytes is induced by Fas-ligand, and the expression of Fas-ligand in angiosarcoma by

immunohistochemistry has also been previously reported.[4] Thus, direct recognition and lysis of tumors by $CD8^+$ cytotoxic T lymphocytes seem to be possible in our cases.

In addition, our PBMC analysis suggests that $CD8^+$ T cells are systemically activated and differentiated in most of the patients analyzed when compared with healthy controls and melanoma patients (Fig. 3*b*), indicating that angiosarcoma patients had more $CD8^+$ cytotoxic T lymphocytes in PBMC. There is a possibility that anti-tumor immunity was activated in angiosarcoma and it seemed not to be immunosuppressive, although we have to highlight the limitations of the *in vitro* ionomycin assay.

Our data suggest that there might be certain tumor immunity against angiosarcoma and if we can activate $CD8^+$ TILs in the tumor-microenvironment and succeed in suppressing tumor growth locally, it might prevent tumor metastases and contribute to a favorable prognosis for patients with angiosarcoma. Furthermore, from our data, even if patients develop distant metastases, it still seems important that higher numbers of $CD8^+$ TILs at primary site contributed to their overall survival. There remains a strong possibility that immunological modification of TILs in the tumor-microenvironment may lead to new treatment strategies of angiosarcoma.

The tumor-infiltrating $CD4^+$ T cells and $FOXP3^+$ T cells were not significantly associated with the prognosis of patients with angiosarcoma in our data, even though suppression of anti-tumor immunity by Tregs is one of the important factors in tumor progression.[22] One possible explanation is that $CD4^+$ helper T cells have functionally different subsets such as T-helper 1 (Th1) cells, T-helper 2 (Th2) cells and immunosuppressive Tregs. Furthermore, all FOXP3-positive cells are not Tregs,[25] because $FOXP3^+$ T cells have functionally different subsets. These subsets have been shown to have opposite influences against tumor-immunity. The balances of these subsets are important for an anti-tumor effect.[34–36] In our data, the production of IFN-γ in $CD4^+$ T cells (ascribed as Th1) significantly increased and percentages of elevated $CD4^+$ $FOXP3^+$ T cells (data not shown) in the peripheral blood of patients with angiosarcoma. Thus, there was a possibility that both anti-tumor Th1 and immunosuppressive Tregs were increased in the tumor microenvironment and were well-balanced.

Donghi *et al.*[37] reported that by analyzing nine patients with the Ki67-index of angiosarcoma, there was a tendency that the patients with the most unfavorable course showed a strong expression of Ki-67, while those with the best outcome only had a slight positive Ki-67 staining. The data from the 30-patient group we analyzed showed no statistically significant differences between the prognosis and Ki67-index of angiosarcoma, but there were tendencies that the groups with a lower Ki67-index had longer survival (data not shown). In this report, we could not include all the stained sections for Ki67-index count due to exclusion criteria. Thus, there is still a possibility that the Ki67-index of tumors is prognostically important for patients with angiosarcoma. Further investigation is required.

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