Human Pathology (2019) **88**, 78–86

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Stromal inflammatory cells are associated with poorer prognosis in primary cutaneous melanoma☆,☆☆

Sook Jung Yun MD, PhD[a], Shujing Liu[b], Meghan Buckley[c], Tao Wang PhD[d], Suna Jin[a], Giorgos Karakousis MD[e], Madalyn G. Peters[e], David E. Elder MB, ChB, FRCPA[b], Phyllis A. Gimotty PhD[c,*], Xiaowei Xu MD, PhD[b,*]

[a]*Department of Dermatology, Chonnam National University Medical School, Gwangju, 61469, Republic of Korea*
[b]*Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*
[c]*Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*
[d]*Office of Biotechnology Products, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, USA*
[e]*Department of Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

Received 16 February 2019; revised 28 March 2019; accepted 29 March 2019

Keywords:
Cutaneous melanoma;
Lymphatic density;
Lymphatic invasion;
Macrophages;
Stromal inflammatory cells;
VEGFC

Summary We observed that non–tumor-infiltrating inflammatory cells are often present in the stroma of melanoma. The role of these stromal inflammatory cells (SIC) in cancer has not been studied. We evaluated the prognostic significance of SIC in 299 patients with vertical growth phase primary melanomas with at least 10 years of clinical follow-up. Lymphatic density and lymphatic invasion in the areas with SIC was quantified. The prognostic significance of these factors was evaluated using univariable and multivariable Cox models for melanoma-specific death and the time to first recurrence. Of the 299 melanomas, 161 exhibited areas with SIC. Percentages of vertical growth phase tumor-infiltrating lymphocytes and radial growth phase regression were significantly higher in cases with SIC compared to those without SIC (P = .005); lymphatic invasion was also detected more frequently in cases with SIC (P = .001). Lymphatic density in SIC areas was higher than that in other areas of the melanomas. Patients with SIC had poorer clinical outcome. Vascular endothelial growth factor-C (VEGFC) staining in a subset of these melanoma patients showed that VEGFC expression in the stromal macrophages was associated with lymphatic invasion in SIC areas. In conclusion, SIC in melanoma is associated with poorer prognosis, and the prognostic effect is partially mediated through induction of lymphangiogenesis with increased lymphatic invasion.

☆ Source of Funding: This work was supported by grants P50-CA-093372 (XX, PG), P30-CA016520 (PG), P50-CA174523 (XX, PG) and PO1CA114046 (XX) from the National Institutes of Health.
☆☆ Conflicts of Interest: None.
* Corresponding authors.
E-mail addresses: pgimotty@mail.med.upenn.edu (P. A. Gimotty), xug@mail.med.upenn.edu (X. Xu).

https://doi.org/10.1016/j.humpath.2019.03.008
0046-8177/

1. Introduction

Inflammatory cells play crucial roles during cancer development, including cancer initiation, invasion, and metastasis [1]. Interactions between cancer cells and immune cells directly or indirectly affect cancer cells as well host response to cancer cells. The abnormal interplay between these cells including direct cell to cell contact and active molecular crosstalk further drives the cancer development. Thus, it is not surprising that density of inflammatory cell infiltrate in cancer has prognostic significance. Many studies have assessed tumor associated inflammatory cells in the context of tumor-infiltrating lymphocytes (TIL) which are defined as lymphocytes that infiltrate or disrupt tumor nests and/or are in direct contact with tumor cells. They can be divided into 3 categories: brisk, non-brisk, and absent [2]. In melanoma, TIL are typically observed during the vertical growth phase (VGP), and is a well-known prognostic factor. Brisk TIL are associated with a better prognosis [2]. Recently, TIL grade was shown to be an independent predictor of sentinel lymph node metastasis and survival [3].

Recent studies from our group demonstrated that lymphatic invasion detected by immunohistochemistry is an independent prognostic factor [4,5]. During the course of these studies, we noticed that increased lymphatic density and lymphatic invasion often occur in the areas with non–tumor-infiltrating stromal inflammatory cells (SIC). In contrast to TIL, SIC forms distinctive aggregates within or at melanoma invasive edge and these cells do not have direct contact with melanoma cells. The SIC is composed primarily of lymphocytes and macrophages.

Tumor-associated macrophages are believed to play an important role in malignancies and they are primarily regarded as pro-tumorigenic, as indicated by the positive effect of macrophages on tumor angiogenesis and growth [6]. In recent years, evidence has accumulated that macrophages are not only critical regulators of angiogenesis, but also crucial participants in lymphangiogenesis, both in inflammatory settings and in tumors [7]. Tumor-associated macrophages may stimulate lymphangiogenesis through either direct secretion of pro-lymphangiogenic factors or by transdifferentiation into lymphatic endothelial cells, actively taking part in the formation of lymphatic vessels as suggested by

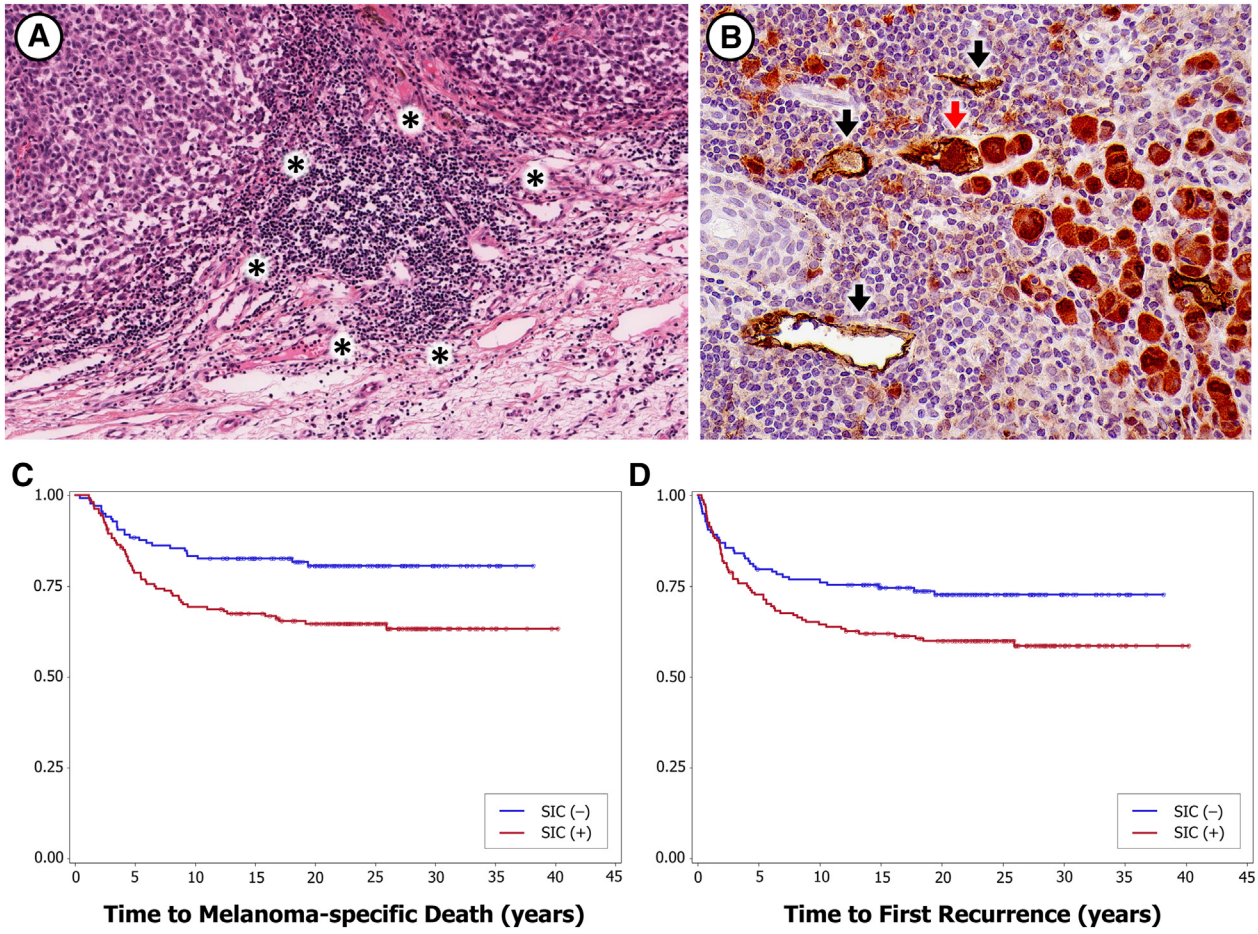

Fig. 1 Histology and clinical significance of stromal inflammatory cells (SIC). A, SIC shows dense non–tumor-infiltrating inflammatory cells (demarcated with asterisks, H&E, original magnification ×100). B, Lymphatic vessels (black arrows) and lymphatic invasion by melanoma cell (red arrow) (S-100 and D2-40 double immunohistochemical stain, original magnification ×400). The Kaplan-Meier survival curves of patients with SIC areas have worse prognosis compared to those without SIC areas, in terms of time to melanoma-specific death (C) and time to first recurrence (D).

Table 1 Patient and tumor characteristics according to SIC status (n = 299)

Characteristic	SIC present (n = 161)		SIC absent (n = 138)		P [a]	All patients (n = 299)	
	n	Percent	n	Percent		n	Percent
Thickness					.711		
≤1.00 mm	62	39%	62	45%		124	41%
1.01-2.00 mm	44	27%	34	25%		78	26%
2.01-4.00 mm	41	25%	30	22%		71	24%
>4.00 mm	14	9%	12	9%		26	9%
Mitotic Rate					.361		
0-0.99	54	34%	56	41%		110	37%
1.00-6.0	60	37%	50	36%		110	37%
>6.00	47	29%	32	23%		79	26%
Ulceration					.138		
Present	38	24%	23	17%		61	20%
Absent	123	76%	115	83%		238	80%
VGP TIL					<.001		
Present	140	87%	70	51%		210	70%
Absent	21	13%	68	49%		89	30%
Gender					.464		
Male	85	53%	67	49%		152	51%
Female	76	47%	71	51%		147	49%
Anatomic site					.438		
Axial	98	61%	90	65%		188	63%
Extremity	63	39%	48	35%		111	37%
Age at diagnosis					.551		
<60 y	108	67%	97	70%		205	69%
≥60 y	53	33%	41	30%		94	31%
RGP regression					.005		
Present	63	39%	33	24%		96	32%
Absent/not available	98	61%	105	76%		203	68%
Satellites					.534		
Present	13	8%	14	10%		27	9%
Absent/unknown	148	92%	124	90%		272	91%
Lymphatic invasion in tumor					.001		
Present	86	53%	48	35%		134	45%
Absent	75	47%	90	65%		165	55%
Lymphatic invasion in SIC					–		
Present	62	39%	–	–		62	20.7%
Absent	99	61%	–	–		99	33.1%
Not applicable	–	–	138	100%		138	46.2%

[a] χ^2 Test.

some authors [8]. In clinical studies, there is a significant correlation between the density of macrophages and lymphatic density in tumor tissues. In human breast cancer, higher numbers of macrophages expressing vascular endothelial growth factor-C (VEGFC) were associated with a higher lymphatic density and lymph node metastasis or lymphatic invasion [9]. However, other studies showed correlation between CD68$^+$/CD163+ macrophages and LYVE-1$^+$ LD/D2-40$^+$ lymphatic invasion, but failed to show association with clinical outcomes in melanoma [10], skin squamous cell carcinoma [11], and Merkel cell carcinoma [12]. The mechanisms underlying the difference is unclear but may be related to methodology or clinical cohorts used.

In this study, we specifically assessed lymphatic density and lymphatic invasion in the areas of SIC using a melanoma cohort with long term clinical follow up and studied the role of VEGFC expression in macrophages in SIC.

2. Materials and methods

2.1. Patients

The study included 299 patients seen between 1972 and 1991 at the Pigmented Lesion Clinic of the University of Pennsylvania who all had at least 10 years of follow-up

Table 2 Cox regression models for melanoma-specific death in the melanoma cohort (n = 299)

Characteristic	Univariable models				Reduced multivariable model			
	HR	LCL	UCL	*P*	HR	LCL	UCL	*P*
SIC – present	2.05	1.29	3.26	.003	1.79	1.14	2.80	.011
Thickness >1	8.85	4.26	18.37	<.001	2.67	1.34	5.34	.006
Mitotic Rate ≥1	5.50	2.84	10.67	<.001	2.64	1.30	5.36	.007
Ulceration present	4.82	3.12	7.44	<.001	1.84	1.19	2.85	.006
VGP TIL absent	0.87	0.53	1.41	.563	1.68	1.01	2.79	.046
Male	2.15	1.36	3.38	.001	–	–	–	–
Anatomic Site – axial	3.43	1.93	6.09	<.001	2.20	1.38	3.52	.001
≥60 y at diagnosis	2.53	1.64	3.89	<.001	–	–	–	–
RGP regression present	1.66	1.07	2.56	.023	–	–	–	–
Satellites present	4.27	2.53	7.22	<.001	2.31	1.37	3.88	.002
Lymphatic invasion in tumor	2.84	1.80	4.48	<.001	1.89	1.23	2.91	.004
Lymphatic density in tumor *	1.04	1.02	1.07	0.001	–	–	–	–

* Included as continuous variables.

and paraffin blocks were available for immunohistochemical staining. Patients had VGP primary melanomas and no apparent metastasis at the time of definitive treatment. The study protocol was approved by the Institutional Review Board of the University of Pennsylvania. The primary endpoints were melanoma-specific death and time to first recurrence.

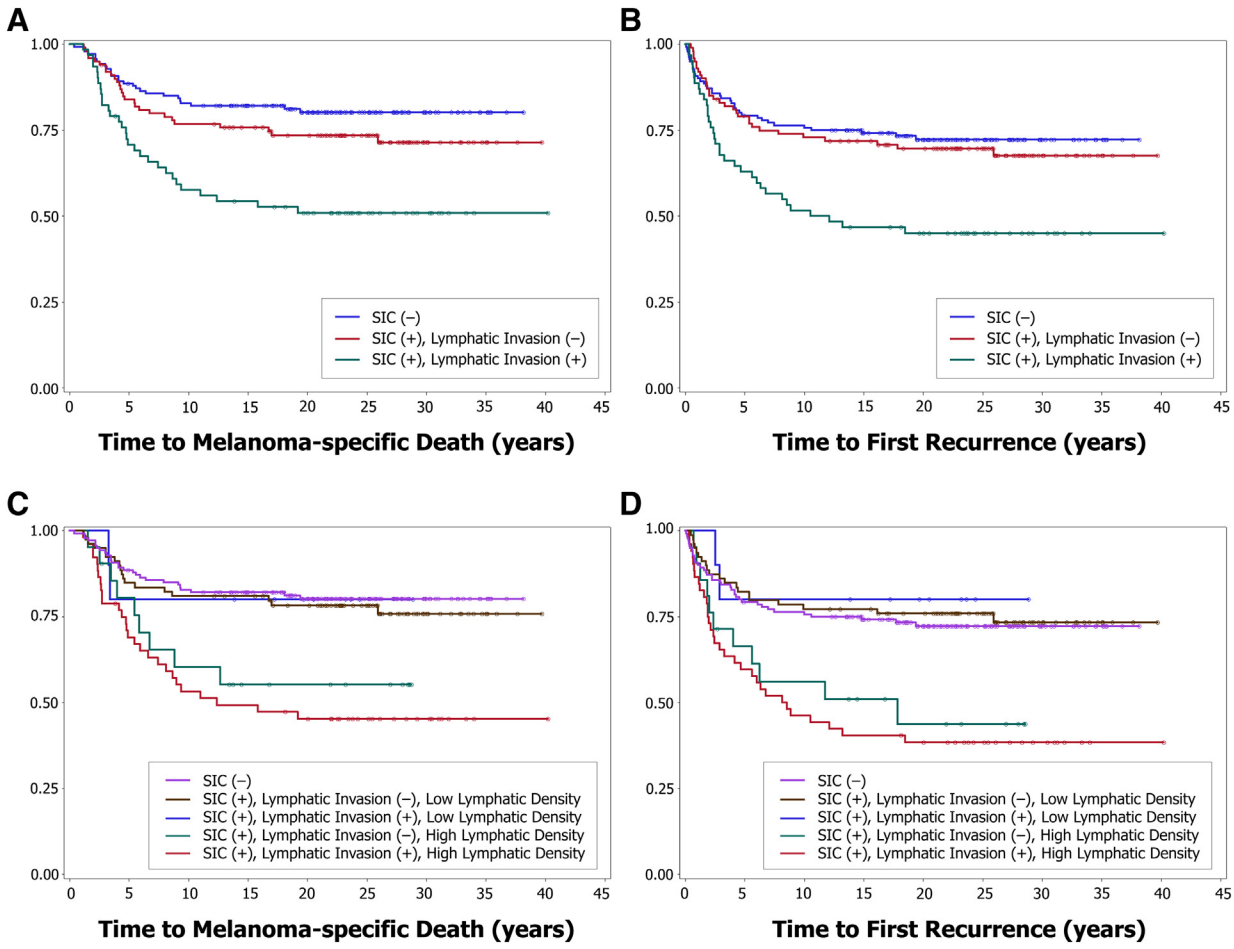

Fig. 2 Kaplan-Meier survival curves for time to melanoma-specific death and time to first recurrence in patients without SIC and those with SIC by lymphatic invasion (A and B) as well as in patients without SIC and those with SIC by lymphatic invasion and lymphatic density (C and D). Patients with SIC with lymphatic invasion (A and B) and patients in the 2 groups with SIC and high lymphatic density in the area of SIC (C and D) have the worst clinical outcomes.

Table 3 Characteristics of SIC patients with macrophage VEGFC by lymphatic invasion status (n = 61)

	Lymphatic invasion in SIC		Lymphatic invasion in SIC		*P* *
	Present (n = 24)		Absent (n = 37)		
	Median	Range	Mean	Range	
Tumor VEGFC	138	5-250	120	5-290	.308
Macrophage VEGFC	77	18-216	37	1-184	.006
Lymphatic density in tumor	9.3	1.0-27.5	5.5	0.0-20.0	.058
Lymphatic density in SIC	15.0	5.0-52.5	16.5	2.5-48.5	.965
Thickness (mm)	1.10	0.31-4.86	1.38	0.55-6.25	.360
Mitotic rate (per mm^2)	1.9	0.0-39.6	2.7	0.0-24.0	.539
Age at diagnosis (y)	53	24-82	53	29-76	.819

* Wilcoxon test.

2.2. Analysis of histopathologic features and stromal inflammatory cells

All slides were originally reviewed for routine histological attributes by 2 pathologists who had no knowledge of patient outcomes. Attributes included tumor thickness, dermal mitotic rate (expressed in mitoses per square millimeter), VGP TIL (classified as present or absent), regression, if present during the invasive or in situ radial growth phase (RGP) adjacent to the VGP, and ulceration. Cut and prepared immunohistochemical stained slides were reviewed independently by 2 dermatopathologists (Yun S.J. and Xu X) who were also blinded to the clinical outcome. An area of SIC was defined as any area with a dense inflammatory cell infiltrate inside or surrounding the melanoma with no direct contact with tumor cells where the greatest dimension of the dense inflammatory infiltrate was more than 0.3 mm (Fig. 1A).

2.3. Assessment of lymphatic density and lymphatic invasion

We performed double immunostaining of lymphatic endothelium, visualized with brown chromogen diaminobenzidine (DAB, DakoCytomation, Carpentaria, CA), using D2-40 antibody (mouse monoclonal, 1:25 dilution, Signet Laboratories, Dedham, MA) and melanoma cells, with Nova Red (Vector Laboratories, Burlingame, CA), using S-100 antibody (rabbit polyclonal, 1:50 dilution, DakoCytomation). Lymphatic density was quantified by counting the number of lymphatic vessels within 1 mm^2 "hot spot" SIC areas. "Hot spots" were discrete areas with easily visible lymphatic vessels positive for D2-40 staining. Lymphatic invasion was defined as S-100 positive melanoma cells within the lumen of D2-40 positive lymphatic vessels (Fig. 1B).

2.4. VEGFC Immunohistochemical staining and quantification

We performed VEGFC immunostaining using VEGFC antibody (mouse monoclonal, 1:100 dilution; Signet Laboratories, Dedham, MA, USA). Staining was performed using a DakoCytomation Autostainer and the EnVision+ horseradish peroxidase DAB system (DakoCytomation) according to the manufacturer's recommendations. Normal mouse serum (1:1000 dilution) was substituted for the primary antibody in each case for use as a negative control. The VEGFC staining intensity in the tumor was graded as follows: 0, negative staining; 1, mild; 2, moderate; and 3, strong staining. Staining results were provided as H-scores, with sum of intensity times positive area percentages. We also counted the number of VEGFC positive macrophages in 1 mm^2 in the SIC areas using "hot spot" method.

Table 4 Characteristics of SIC patients with macrophage VEGFC by lymphatic invasion status (n = 61)

	Lymphatic invasion in SIC		Lymphatic invasion in SIC		*P* *
	Present (n = 24)		Absent (n = 37)		
	Percentage	SE	Percentage	SE	
Ulceration	12.5	6.8	24.3	7.1	.334
VGP TIL	91.7	5.6	86.5	5.6	.694
Male	58.3	10.1	51.4	8.2	.611
Axial	50.0	10.2	62.2	8.0	.430
RGP regression	37.5	9.9	43.2	8.1	.791
Satellites	8.3	5.6	2.7	2.7	.556
Lymphatic invasion in tumor	79.2	8.3	37.8	8.0	.002

* Fisher's exact test.

2.5. Statistical analysis

Patient and tumor characteristics were compared between subjects with and without SIC areas using Pearson's χ^2 statistic or Fisher exact test. Lymphatic density was compared between areas using *t* tests, with Satterthwaite's modification applied in cases of unequal variance. The optimal cut-off point for lymphatic density was defined as the value associated with largest difference between the 2 survival curves, defined according to a range of potential cut-off points. Kaplan-Meier curves were estimated for melanoma-specific death and time to first recurrence, and the log-rank test was used to identify significant differences between curves. Cox proportional hazards regression models were used to obtain hazard ratios (HR; with associated 95% confidence intervals), and to assess whether more specific classification of the SIC areas (ie, in accordance with lymphatic density or lymphatic invasion), and their interaction mediated associations. All *P* values were for 2-sided tests. All *P* values were for 2-sided tests and $P \leq .05$ was considered statistically significant. Analyses were performed using the SAS software package (ver. 9.4; SAS Institute, Cary, NC).

3. Results

3.1. Patients characteristics

The characteristics of the patients according to SIC status are presented in Table 1. Of the 299 total lesions, 161 (53.8%) exhibited SIC and 138 (46.2%) did not. Significantly

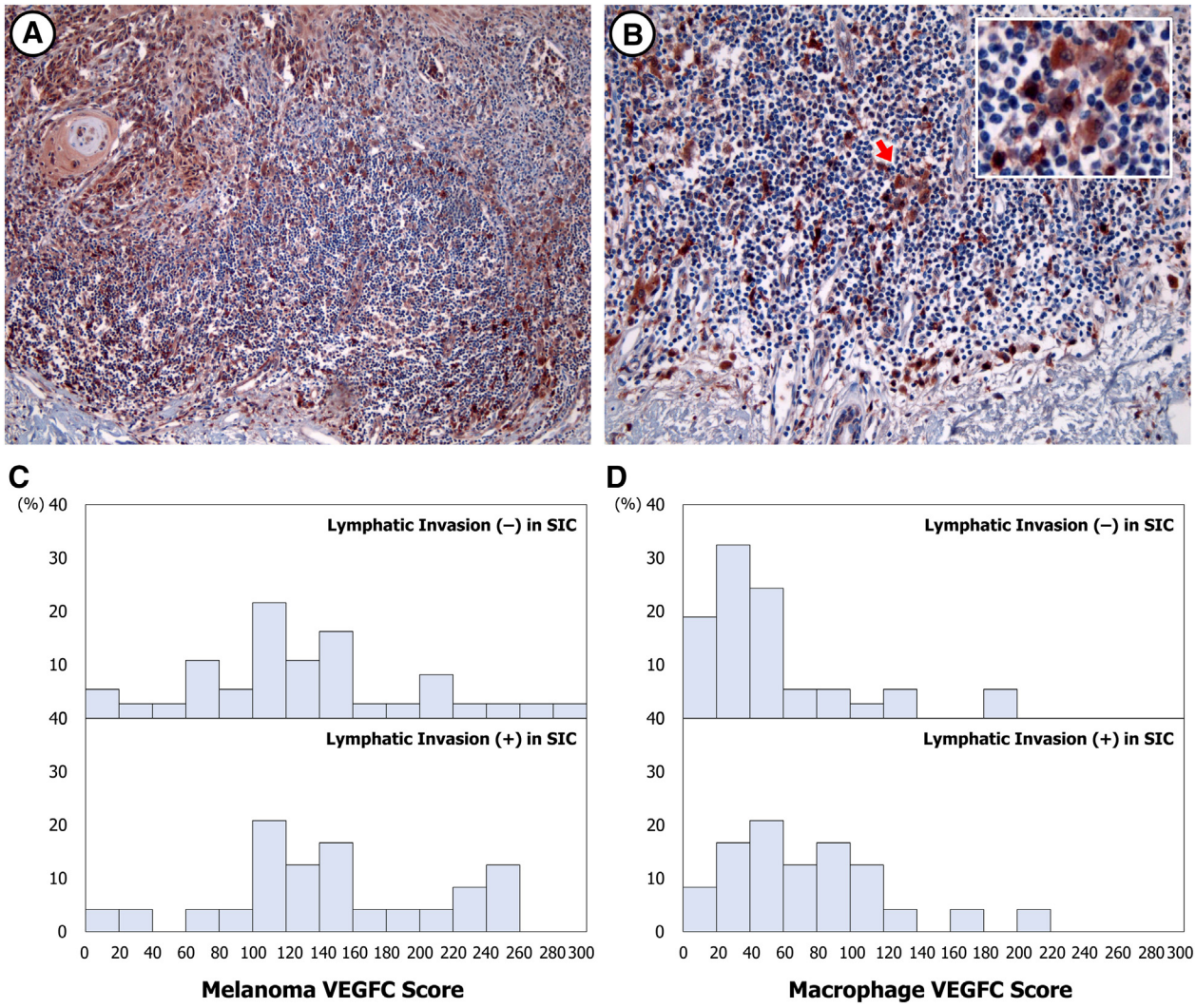

Fig. 3 VEGFC expression in melanoma cells and macrophages in SIC areas. A, VEGFC protein are expressed in melanoma cells (VEGFC immunohistochemical stain, original magnification ×100). B, VEGFC protein are expressed in macrophages (inset shows cytological features of macrophages in red arrow area) (VEGFC immunohistochemical stain, original magnification ×200). C, Distributions of VEGFC expression in tumor cells in SIC areas with and without lymphatic invasion. D, Distributions of VEGFC expression macrophages in SIC areas with and without lymphatic invasion. Macrophage VEGFC expression in the areas SIC is significantly associated with lymphatic invasion.

Table 5 Cox regression models for melanoma-specific death in the macrophage VEGFC subgroup (n = 61)

Characteristic	Univariable models				Reduced multivariable model			
	HR	LCL	UCL	*P*	HR	LCL	UCL	*P*
Thickness >1 mm	7.99	1.86	34.44	.005	6.38	1.06	38.50	.044
Mitotic rate ≥1	7.36	1.71	31.73	.007	11.75	1.95	70.84	.007
Ulceration present	6.42	2.67	15.42	<.001	6.57	2.17	19.88	.001
VGP TIL absent	0.91	0.21	3.90	.895	6.44	1.01	41.16	.049
Male	2.09	0.84	5.18	.113	–	–	–	–
Anatomic site – axial	2.94	1.08	8.05	.036	5.13	1.37	19.19	.015
≥60 y at diagnosis	1.40	0.58	3.37	.460	–	–	–	–
RGR regression present	1.01	0.43	2.40	.984	–	–	–	–
Satellites present	10.17	2.76	37.39	.001	–	–	–	–
Lymphatic invasion in tumor	2.25	0.87	5.82	.093	–	–	–	–
Lymphatic density in tumor *	1.08	1.02	1.14	.007	–	–	–	–
VEGFC score in tumor *	1.00	0.99	1.00	.210	–	–	–	–
Lymphatic invasion in SIC	1.82	0.77	4.29	.171	6.39	2.10	19.49	.001
Lymphatic density in SIC *	1.02	0.98	1.05	.321	–	–	–	–
VEGFC score in macrophage *	1.00	0.99	1.01	.656	1.01	1.00	1.02	.045

* Included as continuous variables.

higher proportions of VGP TIL and RGP regression were present in cases with SIC than cases without SIC ($P < .0001$ and $P = .005$, respectively). Furthermore, lymphatic invasion was more prevalent in cases with SIC than cases without SIC ($P = .001$). Other parameters, such as Breslow thickness, mitotic rate, ulceration, gender, anatomic site, age, and satellites were not significantly different when cases with or without SIC were compared.

3.2. Presence of SIC is associated with poorer prognosis

The presence of SIC in primary melanomas was significantly associated with worse clinical outcome for both melanoma-specific death and time to first recurrence (Fig. 1C and D). We performed univariate and multivariable analysis of melanoma-specific death evaluating SIC and other conventional melanoma microscopic attributes and discovered that the presence of SIC was associated with significantly poorer prognosis (HR = 2.05, $P = .003$ and HR = 1.79, $P = .011$ in the univariable and multivariable models, respectively) (Table 2). Patients were then subdivided into 3 groups based on SIC status and lymphatic invasion status: (1) SIC absent, (2) SIC and lymphatic invasion present, and (3) SIC present but lymphatic invasion absent. For both melanoma-specific death and time to first recurrence, patients with SIC but without lymphatic invasion and patients without SIC had similar survival distributions (Fig. 2A and B) and both had better prognosis than those with SIC and lymphatic invasion present ($P < .001$). Those with areas of SIC were then classified into 4 groups based on lymphatic invasion and lymphatic density. For those with SIC and lymphatic invasion, low lymphatic density was defined to be 5-10.8 (Q1). For those with SIC and no lymphatic invasion, low lymphatic density was defined to be 2.5-23.8 (Q1-Q3). Patients in the 2 groups with SIC and high lymphatic density in the area of SIC had the worst clinical outcomes for both of melanoma-specific death and time to first recurrence (Fig. 2C and D).

3.3. VEGFC-positive macrophages in SIC area are associated with increased lymphatic density and lymphatic invasion

We performed VEGFC immunostaining in 123 of the 299 melanoma patients of which 61 had areas of SIC. Patient and tumor characteristics are shown in Tables 3 and 4. VEGFC expression in melanoma cells was detectable with variable intensity. Interestingly, we found that VEGFC in macrophages in SIC areas was strongly expressed (Fig. 3A and B). We scored the VEGFC expression in the tumor cells and macrophages in the SIC areas and found that tumor VEGFC expression was not associated with LI (Fig. 3C, $P = .308$), but macrophage VEGFC expression in the SIC areas was significantly associated with LI (Fig. 3D, $P = .006$). We performed univariable and multivariable analysis of melanoma-specific death including tumor and macrophage VEGFC expression in SIC as well as other conventional melanoma microscopic attributes. In the multivariable analysis, macrophage VEGFC expression was associated with significantly poorer prognosis (Table 5, $P = .045$).

4. Discussion

This study demonstrates for the first time that SIC in melanomas is associated with worse clinical outcomes. SIC is associated with increased lymphatic density and lymphatic invasion in melanoma. Lymphangiogenesis in SIC areas are

at least partially mediated through VEGFC production by macrophages in the SIC areas.

With increasing importance of immunotherapies for cancer, it is critical to understand the function of tumor associated immune cells. The prognostic value of TIL in primary melanoma has been extensively studied. We are one of the first groups to study the prognostic effect of TIL in melanoma [2]. Recent data reveal an association between TIL grade and patients survival [3,13]. Lower TIL grades are associated with sentinel lymph node metastasis [3]; higher grades are associated with a lower risk of death [13]. In contrast to TIL, SIC does not have direct contact with melanoma cells and presence of SIC is associated with both worse melanoma-associated death and more first recurrence. Erdag et al [14] described 3 types of tumor-infiltrating immune cells in metastatic melanoma; immunotype A cells had no significant infiltration (29% of metastasis); immunotype B (63%) had immune cell infiltration limited to perivascular cuffing; and immunotype C (8%) had diffuse intratumoral immune cell infiltration. Immunotypes A, B, and C had estimated median survival periods of 15, 23 and 130 months in the study, respectively. SIC is distinctively different from immunotype B cells since SIC is often located in the periphery of melanomas. Nevertheless, intratumoral SIC areas are similar to the immunotype B cells as described in the prior publication. Others and our results suggest that location of immune cells is of prognostic value in metastatic melanoma.

Tumor lymphangiogenesis in melanomas has been widely studied and represents an accepted prognostic indicator [15,16]. Our result indicates that lymphangiogenesis is more prevalent in SIC areas. This is supported by prior reports that the highest lymphatic density is observed in melanomas with dense peritumoral inflammatory infiltrates [17]. We previously demonstrated that lymphatic invasion detected by immunohistochemistry was an independent poor prognostic factor in primary cutaneous melanomas [5,18,19]. Current study showed that the effect of SIC is also associated with presence of lymphatic invasion.

Tumor-associated macrophages play a key role in angiogenesis and lymphangiogenesis [7,20]. Macrophage density is associated with poor prognosis in several types of human cancer [20]. Macrophage infiltration is associated with angiogenesis in cutaneous melanoma; the numbers of macrophages and microvessels increases significantly commensurate with increasing tumor depth and tumor angiogenesis [21,22]. Macrophages play a role in lymphangiogenesis, not only through paracrine effects but also by differentiating into lymphatic endothelial cell progenitors that structurally contribute to the growing vasculature [7]. Similar to our study, it has been shown that lymphatic invasion in melanomas was associated with high macrophage counts [10]. In our study, VEGFC expression was strongly positive in macrophages, whereas VEGFC expression in melanoma cells was generally mild to moderate. We also demonstrated that lymphatic invasion in melanoma is not associated with VEGFC expression in the tumor but with VEGFC expression in the macrophages in SIC areas. These results suggest that the effect of SIC may be related to the VEGFC secretion by macrophages in these areas.

In conclusion, our study indicates that SIC is associated with a poor prognosis in melanoma patients. The effect of SIC is associated with increased lymphatic density and lymphatic invasion in these areas. VEGFC-positive macrophages are frequent in SIC areas, and VEGFC produced by macrophages may contribute to increased lymphangiogenesis and lymphatic invasion in SIC areas. Our results suggest that SIC is a new potential biomarker for melanoma progression. In addition, future drugs targeting macrophages and anti-lymphangiogenesis might provide a novel strategy for melanoma treatment.

Acknowledgements

We would like to thank Dr. Clark W. H. Jr for reading the original H&E slides.

References

[1] Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883-99.
[2] Clark Jr WH, Elder DE, Dt Guerry, et al. Model predicting survival in stage I melanoma based on tumor progression. J Natl Cancer Inst 1989; 81:1893-904.
[3] Azimi F, Scolyer RA, Rumcheva P, et al. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. J Clin Oncol 2012;30: 2678-83.
[4] Xu X, Gimotty PA, Guerry D, et al. Lymphatic invasion revealed by multispectral imaging is common in primary melanomas and associates with prognosis. Hum Pathol 2008;39:901-9.
[5] Xu X, Chen L, Guerry D, et al. Lymphatic invasion is independently prognostic of metastasis in primary cutaneous melanoma. Clin Cancer Res 2012;18:229-37.
[6] Yang L, Zhang Y. Tumor-associated macrophages: from basic research to clinical application. J Hematol Oncol 2017;10:58.
[7] Ran S, Montgomery KE. Macrophage-mediated lymphangiogenesis: the emerging role of macrophages as lymphatic endothelial progenitors. Cancers (Basel) 2012;4:618-57.
[8] Kerjaschki D. The crucial role of macrophages in lymphangiogenesis. J Clin Invest 2005;115:2316-9.
[9] Ding M, Fu X, Tan H, Wang R, Chen Z, Ding S. The effect of vascular endothelial growth factor C expression in tumor-associated macrophages on lymphangiogenesis and lymphatic metastasis in breast cancer. Mol Med Rep 2012;6:1023-9.
[10] Storr SJ, Safuan S, Mitra A, et al. Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. Mod Pathol 2012;25:493-504.
[11] Moussai D, Mitsui H, Pettersen JS, et al. The human cutaneous squamous cell carcinoma microenvironment is characterized by increased lymphatic density and enhanced expression of macrophage-derived VEGF-C. J Invest Dermatol 2011;131:229-36.
[12] Werchau S, Toberer F, Enk A, Dammann R, Helmbold P. Merkel cell carcinoma induces lymphatic microvessel formation. J Am Acad Dermatol 2012;67:215-25.

[13] Thomas NE, Busam KJ, From L, et al. Tumor-infiltrating lymphocyte grade in primary melanomas is independently associated with melanoma-specific survival in the population-based genes, environment and melanoma study. J Clin Oncol 2013;31:4252-9.
[14] Erdag G, Schaefer JT, Smolkin ME, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. Cancer Res 2012;72:1070-80.
[15] Rinderknecht M, Detmar M. Tumor lymphangiogenesis and melanoma metastasis. J Cell Physiol 2008;216:347-54.
[16] Pasquali S, van der Ploeg AP, Mocellin S, Stretch JR, Thompson JF, Scolyer RA. Lymphatic biomarkers in primary melanomas as predictors of regional lymph node metastasis and patient outcomes. Pigment Cell Melanoma Res 2013;26:326-37.
[17] Toader MP, Taranu T, Toader S, Chirana A, Taranu T. Correlation between lymphatic vessel density and microvessel density in cutaneous malignant melanoma. Romanian J Morphol Embryol 2014;55:141-5.
[18] Yun SJ, Gimotty PA, Hwang WT, et al. High lymphatic vessel density and lymphatic invasion underlie the adverse prognostic effect of radial growth phase regression in melanoma. Am J Surg Pathol 2011;35:235-42.
[19] Abraham RM, Karakousis G, Acs G, et al. Lymphatic invasion predicts aggressive behavior in melanocytic tumors of uncertain malignant potential (MELTUMP). Am J Surg Pathol 2013;37:669-75.
[20] Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. Front Physiol 2014;5:75.
[21] Kiss J, Timar J, Somlai B, et al. Association of microvessel density with infiltrating cells in human cutaneous malignant melanoma. Pathol Oncol Res 2007;13:21-31.
[22] Torisu H, Ono M, Kiryu H, et al. Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. Int J Cancer 2000;85:182-8.