The clinicopathological impact of tumor-associated macrophages in patients with cutaneous malignant melanoma

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Running title: TAMs in melanoma

Funding: The authors have no funding sources to disclose.

Synopsis for Table of Contents: CD68/pSTAT1 expression (indicating M1 macrophages) was rarely detected in patients with cutaneous malignant melanoma, and high CD68/c-Maf expression (indicating M2 macrophages) was a predictor of worse prognosis.

Abstract

Background: Tumor-associated macrophages (TAMs) are an immune component of the cutaneous malignant melanoma (CMM) microenvironment and affect tumor growth. TAMs can polarize into different phenotypes, i.e., pro-inflammatory M1 and anti-inflammatory M2 macrophages. However, the role of the macrophage phenotype in CMM remains unclear.

Methods: We examined 88 patients with CMM. Tissue microarrays were constructed, and the density of M1 and M2 macrophages was analyzed by immunohistochemistry. Immune cells co-expressing CD68 and pSTAT1 were considered M1 macrophages, whereas those co-expressing CD68 and c-Maf were defined as M2 macrophages. These TAMs were counted, and the relationships between the density of M1 and M2 macrophages and clinicopathological factors including prognosis were investigated.

Results: The CD68/c-Maf score ranged from 0 to 34 (median: 5.5). The patients were divided based on the median score into the CD68/c-Maf high (≥ 5.5) and low (< 5.5) expression groups. Univariate and multivariate analyses revealed that CD68/c-Maf expression was an independent predictive factor for progression-free survival and independent prognostic factor for overall survival. CD68/pSTAT1 expression was found in only two patients.

Conclusion: We suggest that CD68/pSTAT1 co-expression is rarely observed in patients with CMM, and high CD68/c-Maf expression is a predictor of worse prognosis in these patients.

KEYWORDS: cutaneous malignant melanoma, tumor-associated macrophage (TAM), pSTAT1, c-Maf, prognosis

1. INTRODUCTION

Cutaneous malignant melanoma (CMM) is the deadliest type of skin cancer, and the number of CMM cases is increasing worldwide, including Japan [1-3]. CMM mortality is associated with local invasion and metastasis development, and CMM accounts for up to 80% of skin-related deaths [4]. Although recent advances in neoadjuvant immunotherapy, chemotherapy, and targeted therapies have improved patient prognosis, some patients are resistant against these therapies, or achieve only temporary improvement and eventually develop treatment resistance. Therefore, it is necessary to identify patients at high risk of rapid tumor progression and/or developing resistance to therapy.

Tumor cells interact with surrounding stromal cells via complex mechanisms, and together these cells make up the tumor microenvironment (TME) [5]. Within the last decade, the TME has been shown to be important for the proliferation, invasion, metastasis, and treatment resistance of tumor cells [6,7]. Stromal cells include fibroblasts, vascular cells, and immune cells [7,8]. Common immune cells, including lymphocytes, neutrophils, and monocytes/macrophages, also make up the tumor microenvironment [7,8]. Macrophages infiltrating the TME are defined as tumor-associated macrophages (TAMs)[9,10]. TAMs can polarize into different phenotypes, i.e., pro-inflammatory M1 macrophages (classical type) and anti-inflammatory M2 macrophages (alternative type)[11,12]. Both phenotypes are involved in tumor-related inflammation. On the other hand, M1 macrophages destroy tumor cells, whereas M2 macrophages promote angiogenesis, wound healing, and tumor growth [11,12]. In the absence of M1 macrophage-orienting signals, M2 macrophages promote tumor cell

proliferation in vitro and in preclinical models. Evaluation of distinct protumor and antitumor macrophage subsets is a challenging research topic [13,14]. TAMs are the most abundant leukocytes in the CMM [15]. To understand CMM progression, metastasis, and treatment resistance, it is important and useful to distinguish M1 and M2 macrophages in the TME and evaluate them separately.

The aims of this study are to explore the density of M1 and M2 macrophages in CMM using modified immunohistochemical analyses and to investigate the association of this density with clinicopathological factors including prognosis. In addition, programmed death ligand 1 (PD-L1) expression was examined in tumor cells.

2. PATIENTS AND METHODS

2.1 Patients

A retrospective review of a prospectively maintained surgical database was performed to identify patients who underwent primary CMM resection from 2008 to 2017 at Iwate Medical University Hospital. The histopathological diagnosis was reclassified according to the eighth edition of the TNM Classification of the Union for International Cancer Control and the World Health Organization classification of Skin Tumours [16,17]. Patients were excluded from the analysis if they underwent chemotherapy or radiotherapy before surgery, had xeroderma pigmentosum, or had incomplete follow-up data. Finally, 88 patients with CMM were examined. Patient survival was confirmed via medical records and telephone interviews. The end of the follow-up period was May 2021 (median

follow-up period: 1951 days; minimum–maximum: 110–4472 days). The associations between clinicopathological characteristics and immunohistochemical findings were investigated in all 88 patients. This study was approved by the Institutional Review Board of Iwate Medical University (approval no. MH2021-105) and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was waived because this study was retrospective, the patient data remained anonymous, and an opt-out approach was used.

2.2 Evaluation of the mitotic count

The mitotic count was calculated simultaneously by three pathologists (TA, MO, and NY). Whole tissue sections (4 μm thick) were stained with hematoxylin and eosin. In accordance with current AJCC recommendations and Hale's literature, the tumor area with the greatest mitotic activity was identified (the "hotspot")[18,19]. After counting the number of mitotic figures in this field, the count was extended to adjacent fields until a total area of 1 mm^2 was assessed. Each microscope used was calibrated to determine the number of 40× fields equivalent to 1 mm^2.

2.3 Tissue microarray (TMA) construction

The TMAs were created using a manual tissue array (Azumaya Co, Tokyo, Japan). We selected the invasive front of the CMM for immunohistochemical analysis. The selected areas contained massive infiltration of immune cells. Tissue cores (5 mm) were collected from each targeted lesion and placed

into a recipient block containing 12 cores, comprising 11 melanoma tissue cores and 1 control tissue (mucosa of the appendix) core. After construction, 3-µm-thick sections were cut and stained with hematoxylin and eosin on the initial slides to verify the histological diagnosis. Sequential sections were cut from the TMA block for immunohistochemical staining.

2.4 Immunohistochemistry

Blocks were sequentially sectioned at a thickness of 3 µm. Antibodies targeting CD68 (mouse monoclonal, clone PG-M1; Dako, Glostrup, Denmark), phosphorylated signal transducer and activator of transcription 1 (pSTAT1; rabbit monoclonal, clone, 58D6; Cell Signaling Technology, Danvers, MA, USA), c-macrophage activating factor (c-Maf; rabbit monoclonal, clone EPR16484; Abcam, Cambridge, UK) and programmed death ligand 1 (PD-L1, clone 28-8; Dako, Glostrup, Denmark) were used for the immunohistochemical analyses. Staining was performed using the Dako Envision+ system with dextran polymers conjugated to horseradish peroxidase (Dako), as described previously [11]. Sections were stained with anti-CD68 antibodies for 30 min at room temperature using the Vina Green Chromogen Kit (BioCare Medical, Pacheco, CA), producing green staining. Antigen retrieval was performed by heat treatment for 45 min using HIER T-EDTA Buffer (pH 9.0; Dako). After incubation, the tissue sections were reacted with pSTAT1- or c-Maf-specific reagents using dextran polymers conjugated to horseradish peroxidase (Dako) overnight at 4°C, producing green staining. After washing in Wash Buffer (Dako) for 3 min, the sections were counterstained with

hematoxylin. For PD-L1 immunohistochemistry, 3-μm-thick tissue sections were placed on charged slides, dried, and heated at 42°C for 180 min. After deparaffinization and rehydration, the sections were demelanized in 15% hydrogen peroxide at room temperature for 120 min, heated in Envision FLEX target retrieval solution (pH 6.0; Dako) for 20 min, and washed twice for 5 min in phosphate-buffered saline. Hydrogen peroxide (3%) was used to block endogenous peroxidase activity for 5 min. Immunohistochemistry was performed using the DAKO Envision+ system. The specimens were heated in citrate buffer (pH 6.0) for 20 min each using PT Link (Dako). The antigen–antibody reactions were visualized using an enhanced polymer-based detection system. Hematoxylin was used as the counterstain. Sections of appendix were used as the positive controls. The antibodies used in this study are listed in Supplementary Table.

2.5 Assessment of the immunohistochemical results

At first, we examined the immunohistochemical expression of CD68, pSTAT1, c-Maf, and PD-L1 using whole tissue sections to evaluate the heterogeneous expression of these markers. Only nuclear expression of pSTAT1 and c-Maf was considered positive, whereas only cytoplasmic expression of CD68 was regarded as positive. Heterogeneous expression was found within melanoma tissue to some extent. Therefore, 10 high-power fields (HPFs) within the TMAs were evaluated to avoid differences in the heterogeneous positivity of the immune cells by visual assessment. Briefly, we examined areas of very dense CD68 expression and selected 10 representative HPFs. Next, the

pSTAT1 or c-Maf expression status was observed in the same selected fields as much as possible. In this situation, we compared CD68-expressing cells with pSTAT1-expressing cells in sequential section slides, and cells expressing both CD68 and pSTAT1 were counted in 10 HPFs. The cells expressing both cytoplastic CD68 and nuclear pSTAT1 were defined as M1 macrophages (CD68/pSTAT1 positive) (Figure 1a–1c). We also compared CD68-expressing cells with c-Maf-expressing cells in sequential section slides, and the cells expressing both CD68 and c-Maf were counted in 10 HPFs. The cells expressing both cytoplastic CD68 and nuclear c-Maf were defined as M2 macrophages (CD68/c-Maf positive) (Figure 1d–1f). Inflammatory cells were carefully excluded from the analysis. The median score among 10 HPFs was determined for each case. In addition, the sections showing greater than 1% immunohistochemical expression of PD-L1 in the tumor were considered PD-L1 positive. Determination of positive expression was performed by expert diagnostic pathologists (TA, MO, and NY) who were blinded to the study endpoint. If the results among the pathologists were discordant, a consensus was reached via discussion.

2.6 Statistical analysis

Statistical comparisons were performed using Fisher's exact test and the Mann–Whitney U test, as appropriate. Progression-free survival (PFS) and overall survival (OS) were analyzed using the Kaplan–Meier method, and differences in survival were calculated using the log-rank test. PFS was defined as the time from surgery to recurrence, disease progression, or the last follow-up. OS was

defined as the time from surgery to death or the last follow-up. The last follow-up observation was censored if the patient was alive or lost to follow-up. Univariate and multivariate survival analyses were performed using Cox proportional hazards models. All statistical analyses were conducted using JMP Pro 16.1 software (SAS). Results with *p* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 Patient characteristics

The patient characteristics and treatments are summarized in Table 1. Briefly, tumors from 47 males (53.4%) and 41 females (46.6%), with an average age of 76 (range, 31–91) years, were examined. There were 19 (21.6%) patients with CMM at sun-exposure sites. The Clark level of the CMM was II or III in 43 patients. The tumors of 53 (60.2%) patients were classified as stage I or II. Ulcers were present in 38 patients (43.1%). Lymphovascular invasion was detected in 20 patients (22.7%). The mitotic count/mm^2 ranged from 0 to 22 (median: 5); based on the median mitotic count, the tumors were divided into low (< 5/mm^2) and high (> 5/mm^2) mitotic count groups. After surgery, 61 patients received adjuvant chemotherapy. Of the 88 patients, 38 experienced disease progression, and 24 died.

3.2 Infiltration of cells expressing CD68/pSTAT1, CD68/c-Maf, or PD-L1 in the TME

The numbers of CD68/p-STAT1-, CD68/c-Maf-, and PD-L1-positive cells are shown in Table 2.

Briefly, the CD68/pSTAT1 score ranged from 0 to 0.5 (median: 0), and CD68/pSTAT1 co-expression was detected in only two patients. The CD68/c-Maf score ranged from 0 to 34 (median: 5.5), and the patients were divided based on the median score into high (≥ 5.5) and low (< 5.5) expression groups (Table 2B). PD-L1 positivity was detected in 14 (15.9%) patients (Table 2C).

3.3 The relationships between c-Maf expression and clinicopathological characteristics

The relationships between CD68/c-Maf expression and the clinicopathological characteristics are shown in Table 3. The numbers of patients who experienced disease progression and who died were greater in the high than in the low CD68/c-Maf expression groups (p = 0.0013 and p = 0.0017, respectively).

3.4 Univariate and multivariate analyses of progression-free survival and overall survival

The mean follow-up period was 1951 (range, 110–4472) days, during which 38 patients experienced disease progression, and 24 patients died. The 5-year PFS rate in all patients was 58%. The univariate analyses revealed that the pathological stage, presence of ulcers, mitotic count, and CD68/c-Maf expression were significant predictive factors for PFS (Table 4). Figure 2a shows the Kaplan–Meier curve for the 5-year PFS rate according to CD68/c-Maf expression. The rates were 77% and 42% for patients with low and high CD68/c-Maf expression, respectively (p = 0.0027). Multivariate analysis showed that the pathological stage, presence of ulcers, and CD68/c-Maf

expression (HR = 2.58, 95% CI: 1.25–5.34, $p = 0.011$) were independent predictive factors for PFS.

The 5-year OS rate in all patients was 75%. The univariate analyses revealed that pathological stage, presence of ulcers, mitotic count, and CD68/c-Maf expression were significant prognostic factors for OS (Table 5). Figure 2b shows the Kaplan–Meier curve for the 5-year OS rate according to CD68/c-Maf expression. The rates were 86% and 65% for patients with low and high CD68/c-Maf expression, respectively ($p = 0.0033$). Multivariate analysis showed that the pathological stage, mitotic count, and CD68/c-Maf expression (HR = 3.63, 95% CI: 1.32–9.97, $p = 0.012$) were independent prognostic factors for OS.

4. DISCUSSION

Macrophages are activated into M1 (classical type) or M2 (alternative type) phenotypes [11,20]. Immunostaining is often used to examine the roles of these phenotypes. Nitric oxide synthase 2, MRP8-14, Toll-like receptor (TLR) 2, TLR4, CD80, and CD86 are used as M1 markers, whereas CD115, CD163, CD204, CD206, arginase 1, and CD301 are recognized as M2 markers [12,21,22]. However, definitively distinguishing M1 macrophages from M2 macrophages remains difficult [12] because these markers are expressed in other cells, including lymphocytes and leukocytes [14,23]. Moreover, macrophages can express multiple markers simultaneously because of the continuum of phenotypes between M1 and M2 [12,14,23]. Identification of M1 and M2 macrophages relies on accurate selection of markers for differentiation [11]. To overcome this, we used pSTAT1 as an M1-

specific macrophage marker, c-Maf as an M2-specific macrophage marker, and CD68 as a pan-macrophage marker.

STAT1 is upregulated in response to interferon signaling, and its phosphorylated form binds to the promoter region of interferon-stimulated genes [24]. High STAT1 activation promotes M1 polarization of TAMs by increasing the levels of pro-inflammatory cytokines [12,25], suggesting that STAT1 is an M1 marker. TAMs isolated from STAT1-knockout mice failed to induce T-cell responses [12,24,25]. These TAMs lack arginase I activity, which reduces nitric oxide production via inducible nitric oxide synthase (iNOS)[24.26]. Taken together, these data indicate that STAT1 activation in TAMs upregulates iNOS and arginase I activity, resulting in T-cell activation [24]. In our study, co-expression of pSTAT1 and CD68 was found in only two patients, and these two patients showed very low expression of these markers. There are a few reports about the association between M1 macrophages and CMM. Falleni et al. reported that densely infiltrating M1 macrophages, using MRP8-14 as an M1 macrophage marker, in malignant melanoma tumor nests and at the invasive front were associated with distant metastasis and were correlated with a high tumor stage [21]. Furthermore, they found that M1 macrophage accumulation in the tumor stroma was correlated with the Breslow thickness and Clark level, and the abundance of M1 macrophages in malignant melanomas was associated with distant metastasis and was inversely correlated with overall survival, especially when located at the invasive front [21]. On the other hand, Foks et al. found that intratumoral M1 macrophage infiltration was not associated with the skin depth of the melanoma, and they observed

significant positive correlations between the abundance of iNOS-positive cells and survival times [27]. Considering our results, the status of M1 macrophages in CMM is still controversial. One possible reason is the difference in the antibody used in the different studies. We consider that iNOS is not an ideal marker because it is also expressed in melanoma cells. Further studies will be needed.

c-Maf is essential for macrophage self-renewal, but it is also expressed in T cells, including Th2 and Th17 cells [28-30]. Liu et al. identified c-Maf as an essential regulator of immunosuppressive macrophage polarization and showed that c-Maf is predominantly expressed in M2-like macrophages in both mice and humans [28]. Furthermore, inhibition of c-Maf in macrophages results in an M1-like phenotype with reduced immunosuppressive function, and promotes antitumor T-cell immunity, leading to significantly reduced tumor progression [28]. Thus, c-Maf is a core molecule responsible for immunosuppressive macrophage polarization. In our study, we showed for the first time that the number of macrophages expressing both c-Maf and CD68 was correlated with prognosis in patients with CMM, similar to the findings of a previous report in cervical cancer [29]. In other words, patients with high c-Maf expression have worse PFS and OS compared with those with low c-Maf expression. There are several reports on the relationship between the M2 macrophage status and CMM. Jensen et al. reported that melanomas with dense infiltration of CD163-positive macrophages in the tumor stroma were associated with poor OS [31]. Falleni et al. reported that the accumulation of M2 TAMs was associated with poor prognostic indicators and patient survival [21]. Foks et al. reported that the number of CD163-positive macrophages was inversely associated with survival time [27]. Those

reports support our results. Therefore, we suggest that peritumoral infiltration of M2 macrophages is a useful marker to predict the survival of patients with CMM.

Immunotherapy such as cytotoxic T-lymphocytic antigen 4 (CTLA-4)[32-34] and programmed cell death protein 1 (PD-1)[35,36] has been used to treat melanoma. However, 25% of patients with melanoma who show an objective response to PD-1 inhibitors develop resistance [37]. Melanoma specimens resected from patients with refractory metastatic melanoma who were treated with nivolumab, a PD-1 inhibitor used for immunotherapy, exhibited high expression of IL-34 [38]. Importantly, high expression of IL-34 was positively associated with an increased frequency of M2-polarized TAMs [39]. This finding suggests that M2-polarized TAMs may be related to resistance to PD-1 inhibitors in melanoma. Inhibition of c-Maf may help overcome this resistance [25]. Indeed, anti-PD-1 therapy combined with c-Maf inhibition significantly reduced tumor progression [25]. Immunomodulators that specifically target c-Maf in macrophages may be promising treatments because c-Maf is a critical transcription factor in many immune cell subsets [25]. Targeting patients with high numbers of c-Maf-positive macrophages may offer a novel strategy to strengthen current cancer immunotherapies.

This study had some limitations. First, we used TMAs rather than large tissue sections. Although we evaluated the immunohistochemical expression of CD68, pSTAT1, c-Maf, and PD-L1 using whole sections before constructing the TMAs, the issue of heterogeneity cannot be fully resolved. Second, we performed immunohistochemistry using sequential sections rather than the same section.

Although we evaluated the same cells expressing both pSTAT1 and CD68 or both c-Maf and CD68 as much as possible, it may not be perfect.

5. CONCLUSION

In conclusion, our data suggest that CD68/pSTAT1 co-expression is rarely found in patients with CMM, and high CD68/c-Maf expression is a predictor of a worse prognosis in patients with CMM. A shift from the M2 to M1 macrophage phenotype may improve the prognosis of CMM. Further studies are needed.

ACKNOWLEDGEMENTS

The authors would like to thank the members of the Department of Molecular Diagnostic Pathology, Iwate Medical University for their support.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

YA and NY: conception and writing of the manuscript. YA and MS: collection of the clinical data. YA, KI, YK and TT: collection of the samples. YA, NY, MO, NY, RS, and AS: pathological diagnosis and immunohistochemical analyses. NY and TS: revision of the manuscript.

CONSENT FOR PUBLICATION

We guarantee that (a) the work is original, (b) the work has not been and will not be published in whole, or in part, in any other journal, and (c) all of the authors have agreed to the contents of the manuscript in its submitted form.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Review Board of Iwate Medical University (approval no. MH2021-105) and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was waived because this was a retrospective study, the patient data remained anonymous, and an opt-out approach was used.

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FIGURE LEGENDS

Figure 1 Representative images. (a–c) Sequential sections of the same patient with cutaneous melanoma stained with hematoxylin and eosin (a), CD68 (b), or pSTAT1 (c). The cells expressing both cytoplastic CD68 and nuclear pSTAT1 were defined as M1 macrophages (arrow). (d–f) Sequential sections of the same patient with cutaneous melanoma stained with hematoxylin and eosin (d), CD68 (e), or c-Maf (f). The cells expressing both cytoplastic CD68 and nuclear c-Maf were defined as M2 macrophages (arrow).

Figure 2 (a) Kaplan–Meier curve for the 5-year PFS rate according to c-Maf expression. The rates were 77% and 42% for patients with low and high c-Maf expression, respectively (p = 0.0027). (b) Kaplan–Meier curve for the 5-year OS rate according to c-Maf expression. The rates were 86% and

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65% for patients with low and high c-Maf expression, respectively ($p = 0.0033$).

Table 1 Patient characteristics and treatment

Characteristic	Number (%)
Total	88
Sex	
Male	47 (53.4)
Female	41 (46.6)
Age, median [range], years	76 [31–91]
Location	
Sun exposure site	19 (21.6)
Non-sun exposure site	69 (78.4)
Tumor thickness, median [range], mm	3.75 [0.41–26]
Clark level	
II or III	43 (48.9)
IV or V	45 (51.1)
Pathological stage	
I or II	53 (60.2)
III or IV	35 (39.8)
Presence of ulcers	38 (43.1)
Lymphovascular invasion	20 (22.7)
Perineural invasion	3 (3.4)
Solar elastosis	14 (15.9)
Mitotic count, median [range]	5 [0–22]
Underwent adjuvant chemotherapy	61 (69.3)
Progression	38 (43.2)
Death	24 (27.3)
Observation period, median [range], days, PFS	1546 [33–4472]
Observation period, median [range], days, OS	1951 [110–4472]

PFS, progression-free survival; OS, overall survival.

Table 2 CD68/pSTAT1, CD68/c-Maf, and PD-L1 expression

(A)	Range (median)
CD68/pSTAT1 score	0–0.5 (0)
CD68/pSTAT1 score	0–34 (5.5)

(B)	Number (%)
Low CD68/c-Maf expression	43 (48.9)
High CD68/c-Maf expression	45 (51.1)

(C)	Number (%)
Negative PD-L1 expression	74 (84.1)
Positive PD-L1 expression	14 (15.9)

HPF, high-power field; PD-L1, programmed death ligand 1.

Table 3 The associations between CD68/c-Maf expression and clinicopathological characteristics

Characteristic		Low CD68/c-Maf expression		High CD68/c-Maf expression		*p*-value
Number (%)	88	43		45		
Sex						0.675
Male	43	24	(55.8)	23	(51.1)	
Female	41	19	(44.2)	22	(48.9)	
Age, median [range], years	76 [31–91]	76	[44–91]	74	[31–91]	0.073
Location						0.798
Sun exposure site	19	10	(23.3)	9	(20)	
Non-sun exposure site	69	33	(76.7)	36	(80)	
Tumor thickness [range], mm	3.75 [0.41–26]	3.2	[0.5–15]	3.98	[0.41–26]	0.685
Clark level						0.21
II or III	43	18	(41.9)	25	(55.6)	
IV or V	45	25	(58.1)	20	(44.4)	
Pathological stage						0.197
I or II	53	29	(67.4)	24	(53.3)	
III or IV	35	14	(32.6)	21	(46.7)	
Ulcers						0.138
Negative	50	28	(65.1)	22	(48.9)	
Positive	38	15	(34.9)	23	(51.1)	
Lymphovascular invasion						0.449
Negative	68	35	(81.4)	33	(73.3)	
Positive	20	8	(18.6)	12	(26.7)	
Perineural invasion						0.112
Negative	85	40	(93)	45	(100)	
Positive	3	3	(7)	0	(0)	
Solar elastosis						0.568
Negative	74	35	(81.4)	39	(86.7)	
Positive	14	8	(18.6)	6	(13.3)	
Mitotic rate						0.833
Low	40	19	(44.2)	21	(46.7)	
High	48	24	(55.8)	24	(53.3)	
PD-L1						0.782
Negative	74	37	(86)	37	(82.2)	
Positive	14	6	(14)	8	(17.8)	
Adjuvant chemotherapy						0.36
No	27	11	(25.6)	16	(35.6)	
Yes	61	32	(74.4)	29	(64.4)	

Progression	38	11	(25.6)	27	(60)	0.0013
Death	24	5	(11.6)	19	(42.2)	0.0017

Table 4 Associations between clinicopathological features and progression-free survival according to univariate and multivariate analyses.

	Univariate			Multivariate		
	HR (95% CI)		*p*-value	HR (95% CI)		*p*-value
Sex						
Male	1	(Reference)				
Female	1.03	(0.55–1.95)	0.925			
Age	0.98	(0.96–1)	0.086			
Clark level						
II or III	1	(Reference)				
III or IV	1.36	(0.72–2.56)	0.349			
Pathological stage						
I or II	1	(Reference)		1	(Reference)	
III or IV	3.15	(1.64–6.05)	<0.001	2.56	(1.32–4.96)	0.005
Ulcers						
Negative	1	(Reference)		1	(Reference)	
Positive	3.28	(1.68–6.4)	<0.001	2.3	(1.14–4.66)	0.02
Lymphovascular invasion						
Negative	1	(Reference)				
Positive	1.41	(0.7–2.85)	0.333			
Perineural invasion						
Negative	1	(Reference)				
Positive	1.7	(0.41–7.08)	0.468			
Solar elastosis						
Negative	1	(Reference)				
Positive	0.69	(0.24–1.95)	0.484			
Mitotic count						
Low	1	(Reference)		1	(Reference)	
High	2.32	(1.17–4.6)	0.016	1.98	(0.97–4.06)	0.061
CD68/c-Maf expression						
Low	1	(Reference)		1	(Reference)	
High	2.8	(1.39–5.65)	0.004	2.58	(1.25–5.34)	0.011
PD-L1 expression						
Negative	1	(Reference)				
Positive	0.98	(0.41–2.36)	0.97			
Adjuvant chemotherapy						
No	1	(Reference)				
Yes	0.9	(0.44–1.88)	0.485			

HR, hazard ratio; CI, confidence interval.

Table 5 Associations between clinicopathological features and overall survival according to univariate and multivariate analyses.

	Univariate			Multivariate		
	HR (95% CI)		*p*-value	HR (95% CI)		*p*-value
Sex						
Male	1	(Reference)				
Female	0.98	(0.44–2.2)	0.967			
Age	1	(0.97–1.03)	0.82			
Clark level						
II or III	1	(Reference)				
IV or V	1.15	(0.52–2.57)	0.729			
Pathological stage						
I or II	1	(Reference)		1	(Reference)	
III or IV	3.74	(1.6–8.76)	0.002	2.96	(1.25–6.99)	0.013
Ulcers						
Negative	1	(Reference)		1	(Reference)	
Positive	3.75	(1.55–9.05)	0.003	2.18	(0.88–5.36)	0.09
Lymphovascular invasion						
Negative	1	(Reference)				
Positive	1.16	(0.46–2.92)	0.754			
Perineural invasion						
Negative	1	(Reference)				
Positive	1.36	(0.18–10.12)	0.762			
Solar elastosis						
Negative	1	(Reference)				
Positive	0.6	(0.14–2.55)	0.484			
Mitotic count						
Low	1	(Reference)		1	(Reference)	
High	3.71	(1.38–9.96)	0.009	3.46	(1.26–9.49)	0.016
CD68/c-Maf expression						
Low	1	(Reference)		1	(Reference)	
High	3.92	(1.46–10.51)	0.007	3.63	(1.32–9.97)	0.012
PD-L1 expression						
Negative	1	(Reference)				
Positive	0.75	(0.22–2.51)	0.637			
Adjuvant chemotherapy						
No	1	(Reference)				
Yes	0.65	(0.28–1.53)	0.321			

HR, hazard ratio; CI, confidence interval.

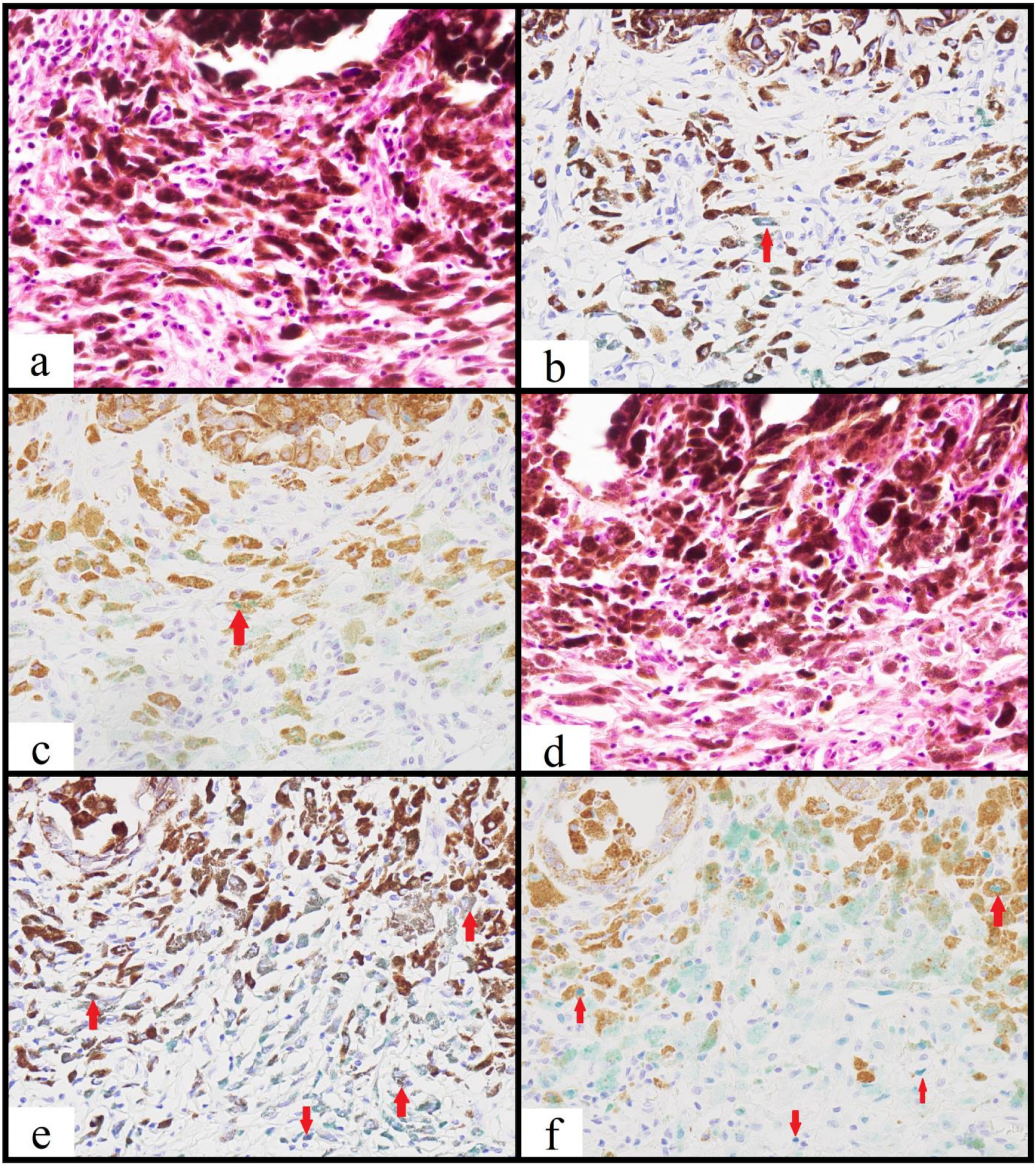
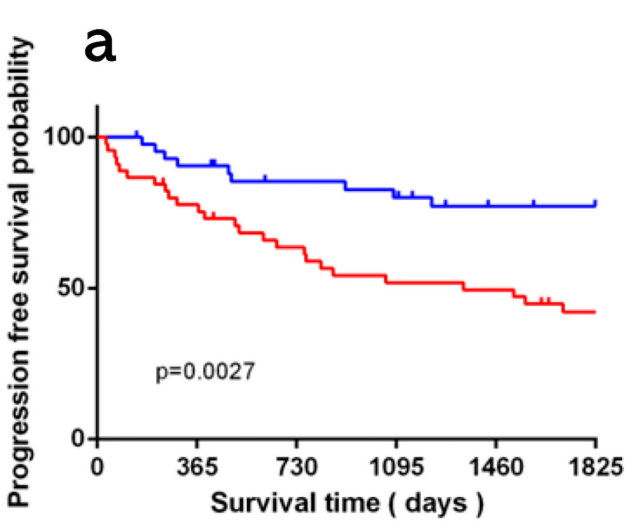
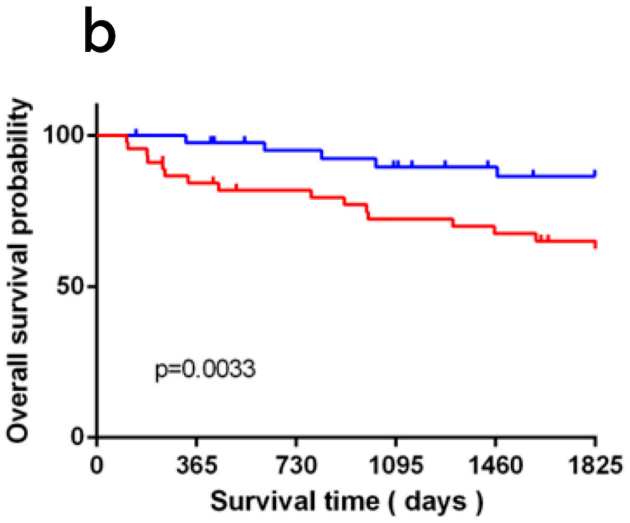


Figure 1



Number at risk						
Low CD68 / c-Maf	43	39	33	30	25	23
High CD68 / c-Maf	45	35	28	23	22	17



Number at risk						
Low CD68 / c-Maf	43	42	37	33	29	26
High CD68 / c-Maf	45	38	35	31	29	26

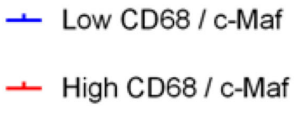


Figure 2

Supplementary table 1

Antibody	Clone	Source	Dilution	Treatment
CD68	PG-M1	Dako	Ready to use	None
pSTAT1	58D6	CST	1:50	Heat retrieval (pH 9.0)
c-Maf	EPR16484	Abcam	1:100	Heat retrieval (pH 9.0)
PD-L1	28-8	Dako	Ready to use	Heat retrieval (pH 6.1)

pSTAT1, phospho-Signal Transducer and Activator of Transcription 1; c-Maf, c-Macrophage activation factor; PD-L1, programmed death ligand 1