

Original Article

Maf expression in human macrophages and lymph node sinus macrophages in patients with esophageal cancer

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The large Maf transcription factors are expressed in immune cells including macrophages and lymphocytes. To investigate the distribution of Maf expression in human organs, immunostaining for Maf was performed using sections of several human organs. High Maf expression was seen in the nucleus of macrophages in the gastrointestinal tract and lymph node sinus macrophages (LySMs). Then, we assessed whether Maf expression in LySMs was correlated with CD169 expression and the clinical prognosis in patients with esophageal cancer. Maf expression was associated with CD169 expression, but Maf expression in LySMs was not associated with the clinical course in patients with esophageal cancer. We determined which cytokines stimulate Maf expression using cultured macrophages. Immunocytochemistry showed that Maf expression was significantly elevated by interferon- γ . These results are the first report of Maf expression in human samples. Maf expression may be a marker for the macrophage population in humans.

Keywords: Maf, macrophage, CD169, esophageal cancer, LySM

INTRODUCTION

Maf (also called c-Maf or MAF transcription factor) is a large Maf transcription factor that binds to the Maf recognition element.¹ Maf is expressed in immune cells including macrophages and lymphocytes.^{2,3} Maf expression is linked to Th2 differentiation in lymphocytes,^{4,5} and the observation that Maf is closely involved in F4/80 expression in macrophages suggests a significant role for Maf in macrophage differentiation.⁶ Maf deficiency induces down-regulation of vascular cell adhesion molecule 1 in fetal liver macrophages and abrogates erythropoiesis in fetal liver.⁷ Maf may also be involved in CCL8 production from sialoadhesin (Siglec-1, CD169)-positive macrophages in mice.⁸

Thus, many studies have investigated Maf, but only a few studies have examined Maf expression in human samples. Following a report of Maf overexpression in angioimmunoblastic T-cell lymphoma,⁹ overexpression of Maf protein was shown in myeloma, T-cell lymphoma, and natural killer/T-cell lymphoma.¹⁰ In a study using a murine model, Maf


overexpression in T lymphocytes induced the development of T-cell lymphoma, suggesting that Maf protein leads to malignant transformation in T lymphocytes.¹¹ In normal tissues, Maf expression is detected in the subpopulation of plasma cells, lymphocytes, and macrophages in hematopoietic organs, although the expression levels are weak.¹⁰ However, Maf expression was examined only in hematopoietic and lymphoid organs. In the present study, we investigated Maf expression in human organs and showed that Maf expression was present in resident macrophages in the gastrointestinal tract and lymph nodes. Maf expression was expressed in lymph node sinus macrophages (LySMs), which are antigen-presenting cells that contribute to anti-viral and anti-cancer immune responses.¹²⁻¹⁴ We previously reported that increased CD169 expression in LySMs was correlated with increased infiltration of lymphocytes into cancer tissues and a better clinical course in several malignant cancers including esophageal cancer.^{15,16} Therefore, here we tested if Maf expression in LySMs was correlated with CD169 expression and the clinical prognosis in patients with esophageal cancer

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using the same lymph node samples.

MATERIALS AND METHODS

Samples

Paraffin-embedded samples of several organs other than gastro-intestinal tracts were prepared from specimens obtained from three autopsy cases at Kumamoto University Hospital. Samples of stomach and small/large intestine were obtained from the non-cancerous part of resected colon cancer samples. Paraffin-embedded lymph node samples were prepared from specimens obtained from 182 patients diagnosed with esophageal cancer between 2005 and 2013 at Kumamoto University Hospital.¹⁶ Written informed consent was obtained from all patients in accordance with protocols of the Kumamoto University Review Board, and the study design was approved by the Kumamoto University Review Board (#1174, #2224). Cancer staging was performed according to the American Joint Committee on Cancer Staging Manual (7th edition). In cases involving lymph node metastasis, only cancer cell-free lymph nodes were used for the analysis.

Immunohistochemistry

Single and double immunohistochemical staining was performed as described previously.^{16,17} In brief, deparaffinized sections were microwave treated in 1 mM EDTA (pH 8.0). Then, sections were incubated with anti-Maf antibody (clone EPR16484, Abcam), anti-CD204 (scavenger receptor A type I and II or macrophage scavenger receptor 1) antibody (clone SRA-E5, Cosmo Bio, Tokyo, Japan), or anti-CD169 antibody (clone HSn 7D2; Santa Cruz Biotechnology, CA, USA). Horseradish peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin antibody (Nichirei, Tokyo, Japan) was used as the secondary antibody. Diaminobenzidine (brown color) and HistoGreen (green color) substrate (#AYS-E109, Cosmo Bio) were used for visualization of positive signals. The specificity of anti-Maf antibody was checked by immunohistochemistry using cell block specimens of RPMI8226 (Maf-positive, gifted from Dr. Yutaka Okuno, Kumamoto University) and MOLT4 (Maf-negative, purchased from JCB cell bank, Osaka, Japan). Scoring of Maf was done according to our previously published study.^{16,18} The immunohistochemical data for CD169 in patients with esophageal cancer were previously published.¹⁶ For scoring of Maf expression, all samples were evaluated microscopically by two pathologists (H. T. and K. O.) who were blinded to the patient's information. The intensity score and the proportion score were determined based on the intensity (score 0; negative, 1; weak, 2; moderate, or 3; strong) or the proportion of the stained area (score 0; negative, 1; 1%-10%, 2; 11%-50%, or 3; >50%), respectively. The Maf score was calculated by adding the intensity (0-3) and the proportion (0-3) scores and ranged from 0 to 6. The average of the total score of the two pathologists was the final score.

Macrophage culture

Peripheral blood mononuclear cells were obtained from healthy volunteer donors who each provided written informed consent for the use of their cells in accordance with the study protocols approved by the Kumamoto University Hospital Review Board (#1169). Monocytes were isolated using RosettSep cocktail (StemCell Tech., Vancouver, Canada), plated in UpCELL culture plates (CellSeed, Tokyo, Japan), and cultured in 2% human serum, 1 ng/mL granulocyte macrophage-colony stimulating factor (WAKO, Tokyo, Japan), and 50 ng/mL macrophage-colony stimulating factor (WAKO) for 7 days to induce macrophage differentiation. Macrophages (2×10^5 /well) were then seeded on glass coverslips in a 12-well plate and stimulated with interleukin (IL)-10 (#093-04651, 10 ng/mL, WAKO), interferon (IFN)- α (#11200-2, 10 ng/mL, R&D Systems, Minneapolis, MN, USA), IFN- γ (#IFG4001, 10 ng/mL, WAKO), or lipopolysaccharide (LPS) (#L2654, 100 ng/mL, Sigma, St. Louis, MO, USA) for 1 day. Cells were fixed with 1% paraformaldehyde and then dried once. Cells were blocked with 1% bovine serum albumin and then stained with anti-Maf antibody. Horseradish peroxidase-labeled anti-rabbit immunoglobulin antibody (Nichirei) was used as the secondary antibody, and diaminobenzidine substrate was used for visualization.

Statistics

JMP10 software (SAS Institute, Chicago, IL, USA) was used for statistical analyses. The cumulative survival rate was compared between two groups using the log-rank test and Wilcoxon test. The median was used as the cut-off value for comparisons between two groups. The Chi square test and Student's *t*-test were also performed. A *p*-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Macrophages in intestines and lymph nodes express Maf protein.

At first, the specificity of anti-Maf antibody was evaluated by immunostaining using cell block specimens of Maf-positive cell line (RPMI8226) and Maf-negative cell line (MOLT4), and the result showed that Maf expression was observed in RPMI8226 and not seen in MOLT4. Then, we tested the immunostaining of Maf and CD204 (a marker for macrophage). Strong Maf expression was detected in the nucleus of resident macrophages in the lamina propria of the stomach, small intestine, and large intestine (colon), and was also seen in LySMs (Figure 1, Table 1). Weak expression was observed in macrophages in the heart, cerebellum, and bone marrow (Table 1).

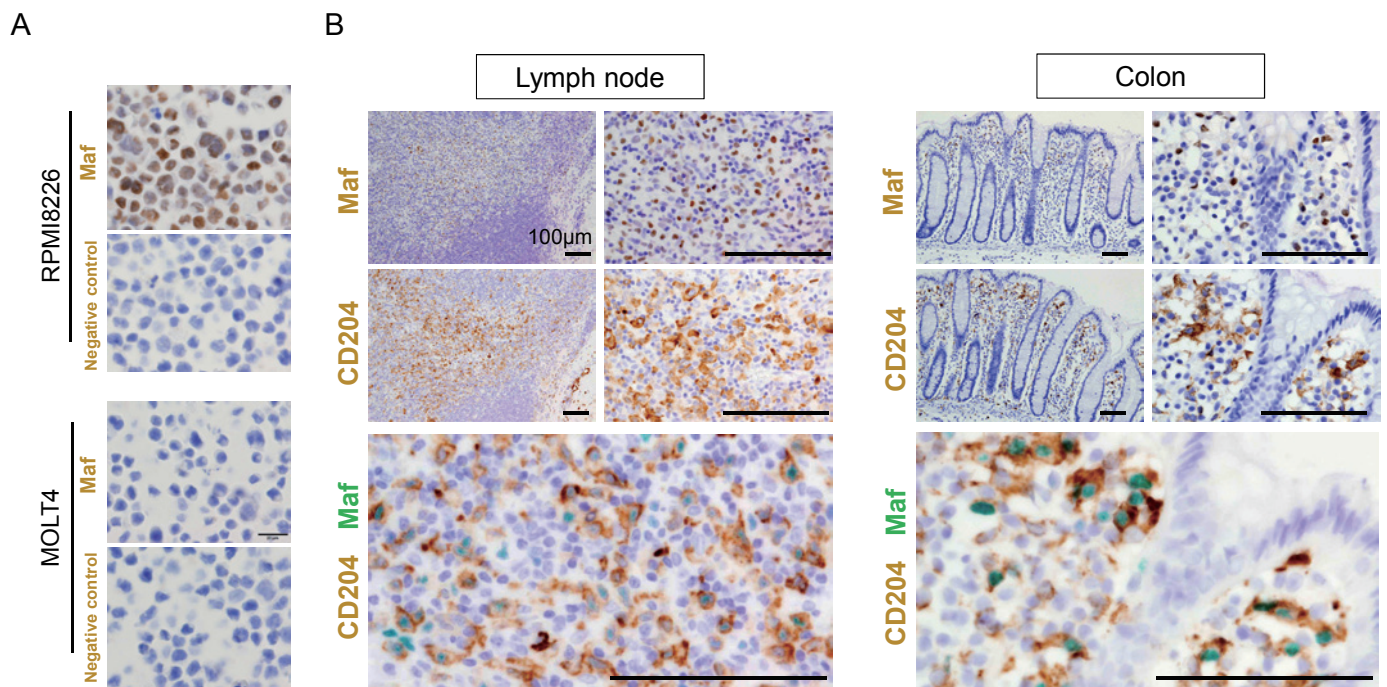


Fig. 1. Immunohistochemistry for Maf. (A) Single immunostaining for Maf was performed on sections of cell blocks of two cell lines (RPMI8226 and MOLT4). Scale bar; 20 μ m. (B) Single immunostaining for Maf and CD204 (a marker for macrophages) and double immunostaining for Maf (green) and CD204 (brown) were performed on sections of lymph nodes and colon. Scale bar; 100 μ m.

Table 1. c-Maf expression in different human organs

Tissues	c-Maf	Tissues	c-Maf
Heart		Thyroid	
	Intermuscular M ϕ		Interstitial M ϕ
Lung		Trachea	
	Alveolar M ϕ		Interstitial M ϕ
Liver		Esophagus	
	Kupffer cells		M ϕ in lamina propria
	M ϕ in portal triads	Stomach	
Kidney			M ϕ in lamina propria
	Interstitial M ϕ	Adrenal	
	Urineriferous tubule cells		Interstitial M ϕ
Spleen		Prostate	
	Red pulp M ϕ		Interstitial M ϕ
	White pulp M ϕ	Aorta	
Thymus		Large intestine	
	M ϕ in cortex		M ϕ in lamina propria
	M ϕ in medulla	Small intestine	
Lymph nodes			M ϕ in lamina propria
	M ϕ in follicles	Cerebrum	
	M ϕ in paracortical areas	Cerebellum	
Pancreas		Bone marrow	
	Interstitial M ϕ		

+, positive, \pm ; weakly positive, -, negative, M ϕ ; macrophage.

Maf expression in LySMs in patients with esophageal cancer.

We previously showed that CD169 expression in LySMs of non-metastatic regional lymph nodes was correlated with better clinical prognosis in patients with esophageal cancer. In the present study, we examined the expression of Maf in the same samples used in the previous study¹⁶ (Figure 2A). The distribution of the final scores is shown in Figure 2B and table 2. Cases with a score >4 were classified as “high” to divide the distribution of final scores. Double immunostaining for CD169 and Maf showed that a portion of Maf-positive LySMs were also positive for CD169 (Figure 2C). Statistical analysis demonstrated that the high-Maf cases were preferentially associated with high-CD169 cases (Figure 2D). However, Maf expression in LySMs was not associated with any clinicopathological factors and the clinical course in patients with esophageal cancer (Figure 2E, Table 2).

Increased expression of Maf protein was seen in IFN-γ-stimulated macrophages.

Next we tested which cytokines stimulate Maf expression in cultured macrophages. Monocytes derived from peripheral blood were differentiated into macrophages and then stimulated with IFN-α, IFN-γ, IL-10, or LPS. Immunocytochemistry showed that Maf expression was significantly elevated by IFN-γ (Figure 3), whereas no significant effect was observed on macrophages treated with IFN-α, IL-10, or LPS.

DISCUSSION

In the present study, we performed double immunostaining for Maf and CD204 (a marker for macrophages), and strong Maf expression was seen in resident macrophages in the human stomach, small/large intestine, and lymph nodes, consistent with previous studies in mice.³ In addition, although Maf plays a critical role in Th differentiation in lymphocytes,^{19,20} no positive signals were seen in lymphocytes in human organs. The sensitivity of immunohistochemistry

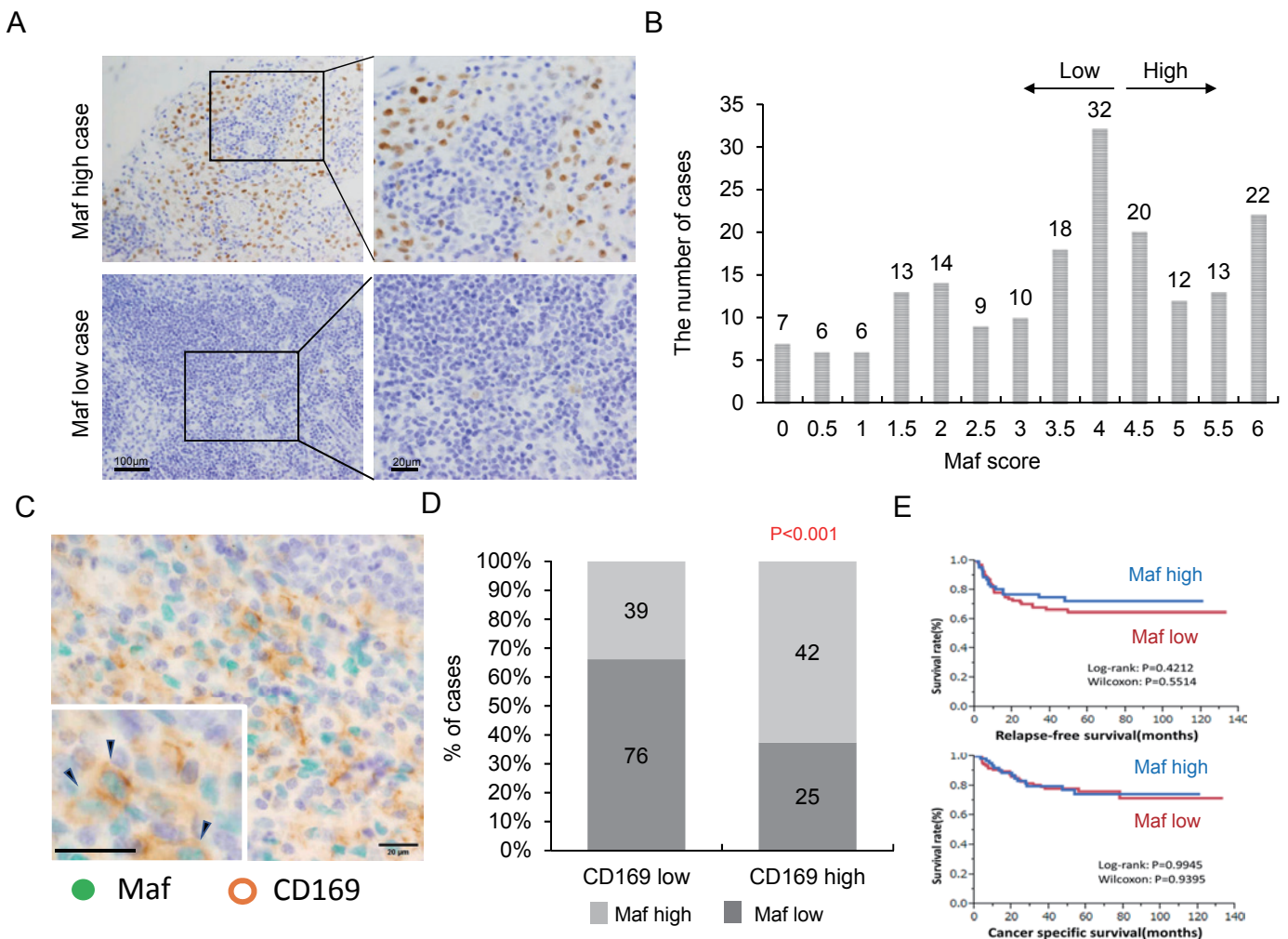


Fig. 2. Maf expression in LySMs of patients with esophageal cancer. (A) Immunostaining of Maf in LySMs of patients with esophageal cancer. Representative results of two cases are presented. (B) Maf expression in LySMs, as indicated by the Maf score. (C) Double immunostaining for Maf (green) and CD169 (brown) is presented. (D) The correlation between Maf and CD169 expression in LySMs was assessed with the Chi-square test. (E) Kaplan-Meier analysis of the Maf score and clinical course.

Table 2. Relationship between expression of Maf and clinicopathological feature

		n= 182	c-Maf expression		
			Low	High	
Mean age (years)			66.94	65.25	N.S.
Gender					
	Male	160	97	53	N.S.
	Female	22	18	4	
Histology					
	SCC	166	102	64	N.S.
	others	16	13	3	
T status					
	pT1	92	59	33	N.S.
	pT2	23	13	10	
	pT3	61	37	24	
	pT4	6	6	0	
Pretreatment					
	Chemotherapy	74	46	28	N.S.
	None	108	69	39	
Stage					
	I	69	43	26	N.S.
	II	63	41	22	
	III	41	27	14	
	IV	9	4	5	
LV invasion					
	Negative	95	60	35	N.S.
	Positive	87	55	32	

SCC: squamous cell carcinoma, LV: lymphovascular. N.S.; statistically not significant.

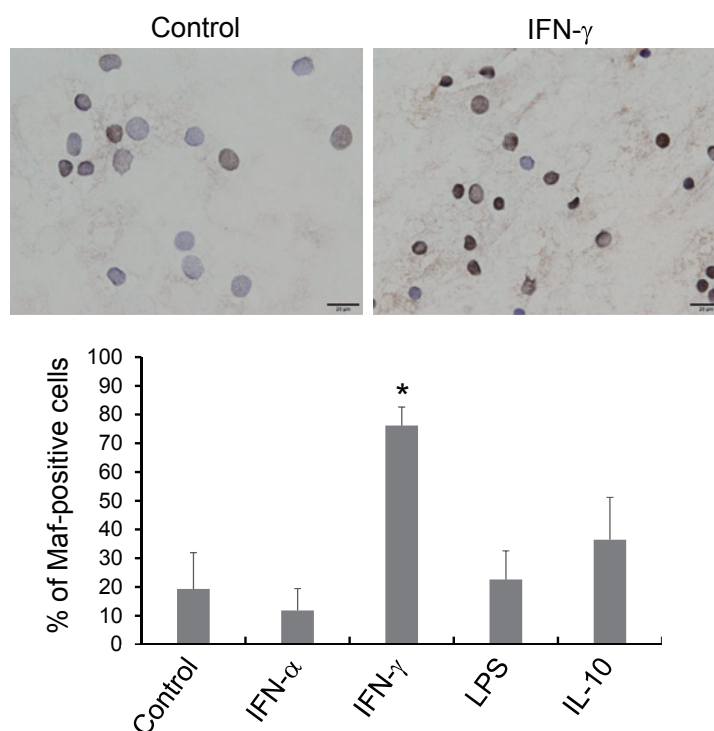


Fig. 3. Immunocytochemistry for Maf. Immunostaining for Maf was performed using cultured macrophages treated with IFN- α , IFN- γ , IL-10, or LPS. Positive signals were seen in the nucleus, and the percent positive cells was calculated (n = 3 per sample).

is generally weaker than that of flow cytometry or PCR, and these technical factors may be the reasons for this discrepancy. Furthermore, the signal intensity of Maf was weaker in autopsy specimens than surgically resected samples including gastro-intestinal tracts and lymph nodes in the present study. The possibility that the discrepancy of fixation period and postmortem alterations between autopsy samples and surgically resected materials influenced the Maf-positive signals cannot be excluded.

Another interesting observation of this study is that a portion of Maf-positive LySMs was negative for CD169, suggesting a complex heterogeneity for LySMs. We previously demonstrated that CD169 expression in human monocyte-derived macrophages was elevated by IFN- α , IFN- β , IFN- γ , and LPS, and the most significant up-regulation was induced by IFN- α and IFN- β .²¹ In the present study, Maf expression was significantly induced by IFN- γ , but not by IFN- α or LPS. We also previously showed that expression of indoleamine 2,3-dioxygenase in human monocyte-derived macrophages was elevated by IFN- α , IFN- β , IFN- γ , and LPS, with the most significant up-regulation induced by IFN- γ .²² Indoleamine 2,3-dioxygenase expression does not overlap with CD169 expression in LySMs, and thus, we suggest that at least two subpopulations of LySMs exist.¹⁶ Resident and exudate macrophages may co-exist in lymph nodes and other organs. In addition, some resident macrophages are considered to originate from yolk sac primitive macrophages, which have self-renewal ability.²³ Fate mapping studies have revealed the heterogeneity of resident macrophages in several organs,^{24,25} although few studies have investigated the heterogeneity of macrophages in lymph nodes. Further studies are necessary to reveal the heterogeneity of macrophages in lymph nodes.

In conclusion, Maf expression was detected in resident macrophages in the gastrointestinal tract and lymph nodes with immunohistochemistry. In patients with esophageal cancer, Maf expression was positively associated with CD169 expression. Maf expression was induced by IFN- γ stimulation of cultured macrophages. These results are the first report of Maf expression in human samples. Maf expression may be a marker for the macrophage subpopulation in humans.

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CONFLICT OF INTEREST

All authors have no financial competing interests to declare.

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