Phospho-STAT5B Expression Is a Prognostic Marker for Merkel Cell Carcinoma

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Abstract. *Background/Aim:* Merkel cell carcinoma (MCC) is an aggressive cutaneous neuroendocrine carcinoma. Although recent reports suggest that tumor-infiltrating leukocytes (TILs), especially CD8⁺ T-cells, contribute to the pathogenesis of MCC, it is difficult for a single Institute with a small number of patients with MCC to determine the threshold number of CD8⁺ cells. Therefore, clearer and easier methods of evaluating prognostic factors of MCC are needed. *Patients and Methods:* In order to identify the prognostic factors of 24 cases of MCC, we employed immunohistochemical staining of phospho-signal transducer and activator of transcription 5B (pSTAT5B), which has been reported to be a prognostic marker for several types of cancers. *Results:* All MCC cases with a good outcome (n=16) expressed pSTAT5B, whereas all MCC cases with a poor outcome (n=8) did not express pSTAT5B. Moreover, we additionally employed immunohistochemical staining of periostin (POSTN) and interleukin-4, as well as sub-populations of TILs (granulysin-bearing cells, regulatory T-cells, CD163⁺ cells, and CD206⁺ cells), and the deposition of matrix metalloproteinase 12 in the lesional skin of patients with MCC. The results suggested that there is no significant difference in stromal factors between MCC cases with a good and those with a poor outcome. *Conclusion:* pSTAT5B expression may be an indicator of positive prognosis in patients with MCC.

Merkel cell carcinoma (MCC) is an aggressive cutaneous neuroendocrine carcinoma that originates either from Merkel

cells or from pluripotent stem cells in the basal layer of the epidermis (1). Recent reports suggested that tumor-infiltrating leukocytes (TILs) contribute to the pathogenesis of MCC, and that TILs might determine the prognosis and tumor-specific survival of patients with MCC (1-3). For example, Paulson *et al.* reported an association between intratumoral CD8⁺ lymphocyte infiltration and MCC-specific survival (2). Although these reports suggested that infiltration by CD8⁺ lymphocytes might be a prognostic factor of MCC and that the immunological background of MCC is important, it is difficult for a single Institute with a small number of patients with MCC to determine the threshold number of CD8⁺ cells to indicate a positive prognosis. Therefore, clearer and easier methods of evaluating prognostic factors of MCC are needed. In this report, we describe 16 cases of MCC with a good outcome, all of which expressed phospho-signal transducer and activator of transcription 5B (pSTAT5), and eight cases of MCC with a poor outcome, none of which expressed pSTAT5.

Materials and Methods

Materials. We used the following antibodies for immunohistochemical staining: mouse monoclonal for human pSTAT5B (Abcam, Tokyo, Japan), CD163 (Novocastra, Milton Keynes, UK), CD206 (LifeSpan BioScience, Seattle, WA, USA); goat polyclonal for human matrix metalloproteinase-12 (MMP12) (R & D Systems, Minneapolis, MN, USA); rabbit polyclonal for human periostin (POSTN; Abcam), interleukin-4 (IL4) (LifeSpan BioScience), CD8 (Dako, Kyoto, Japan), granulysin (R & D Systems), and forkhead box P3 (FOXP3) (Abcam).

Patients, tissue samples and immunohistochemical staining. We collected archival formalin-fixed paraffin-embedded skin specimens from each of 24 patients with primary MCC treated at one of three hospitals in Japan: Tohoku University Hospital, Wakayama Medical University Hospital, or Shiga University of Medical Science Hospital (Table I). All patients provided their informed consent.

We retrospectively divided the MCC cases into four outcome groups: those with spontaneous regression (tumor completely disappeared after biopsy) (25, 26) (excellent outcome); those with no recurrence for at least 3 years (good outcome); those with

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Key Words: Merkel cell carcinoma, pSTAT5, prognostic marker, cancer stroma.

Table I. Summary of characteristics and immunohistochemical staining in 24 cases of Merkel cell carcinoma in this study. Intensity of staining was scored semi-quantitatively as: - : Negative; + : moderate, ++ : intense.

Group	Case	Age (years)/Gender	pSTAT5B	POSTN	MMP12	IL4
Spontaneous regression	1	71/M	++	+	-	+
	2	94/F	++	++	-	-
	3	86/F	++	+	++	-
	4	67/F	++	+	-	-
	5	94/F	++	+	++	+
	6	87/M	++	+	-	+
	7	84/F	+	++	+	+
	8	89/M	++	++	-	-
Without remote metastasis	9	77/M	++	++	++	+
	10	83/F	+	++	-	+
	11	72/F	++	++	+	+
	12	85/F	+	++	+	-
	13	80/M	+	+	+	-
	14	78/M	++	+	+	-
	15	79/F	++	+	+	+
	16	93/F	+	+	-	-
	17	83/M	-	++	++	-
	18	79/F	-	+	-	-
With remote metastasis	19	74/F	-	++	+	++
	20	90/F	-	+	-	-
	21	96/F	-	+	++	+
	22	71/M	-	++	++	+
Died of disease	23	78/F	-	-	+	-
	24	86/M	-	++	+	-

F: Female; M: male; pSTAT5B: Phospho-signal transducer and activator of transcription 5B; POSTN: periostin; MMP12: matrix metalloproteinase 12; IL4: interleukin-4.

recurrence with distant metastasis (poor outcome); or death due to MCC (poor outcome) (Table I). The study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine, Sendai, Japan (2014-1-755). Before the antigen retrieval procedure, 0.3% H₂O₂ in methanol was used to inhibit endogenous peroxidases. Samples from the 24 cases of MCC were processed for single staining of pSTAT5B and developed with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) and its enhancer (Leica Microsystems, Tokyo, Japan), or for single staining of POSTN, IL4, CD163, CD206, CD8, granulysin, FOXP3, or MMP12.

Assessment of immunohistochemical (IHC) staining. The staining of tumor cells, stromal cells, or stromal factors was examined in more than three random representative fields of each section. Immunoreaction for pSTAT5B and FOXP3 was assessed as nuclear staining, and that for CD163, CD206, CD8 and granulysin was assessed for cytoplasmic staining (21, 27). MMP12, IL4 and POSTN staining of cancer stroma (8). Isotype controls for each antibody were used for negative controls. For the evaluation of pSTAT5B staining, tumor samples which contained more than 90% of positively stained nuclei were classed as having intense staining, those with more than 70% as moderate, and fewer than 70% as negative. For POSTN, MMP12 and IL4 staining, staining intensity was evaluated by two dermatopathologists as we previously reported (8).

The intensity of IHC staining was scored on a semi-quantitative scale (-: negative; +: moderate; ++: intense), and the results are

shown in Table I. Data are expressed as the mean $\pm$ standard deviation of the IHC score in each group of MCC.

Statistical methods. For single comparisons between two groups, the Mann-Whitney *U*-test was used. The level of significance was set at $p < 0.05$.

Results

Expression of pSTAT5B on tumor cells in MCC. Since the expression of pSTAT5 is a positive prognostic marker for several solid tumors, such as breast cancer (4, 5), we hypothesized that the expression of pSTAT5B would differ between MCC cases with a good or a poor outcome. In two cases of MCC with spontaneous regression, almost all tumor cells strongly expressed pSTAT5B (Figure 1A). In 14 cases of MCC without recurrence, more than 70% of the tumor cells strongly expressed pSTAT5B (Figure 1B). In contrast, in four cases of MCC with recurrence (Figure 1C) and four cases with death due to MCC (Figure 1D), pSTAT5B was scarcely expressed. The intensity of IHC staining, as scored on the semi-quantitative scale, is shown in Table I.

Expression of POSTN and IL4 in the lesional skin of patients with MCC. Since the expression of POSTN regulates the

Figure 1. The localization of phosphorylated signal transducer and activator of transcription 5B (pSTAT5B) in the nuclei of Merkel cell carcinoma (MCC) cells. Paraffin-embedded tissue samples of the lesional skin of patients with MCC with spontaneous regression (tumor completely disappeared after biopsy: excellent outcome) (A), no recurrence for at least 3 years (good outcome) (B), recurrence with distant metastasis (poor outcome) (C), or death due to MCC (poor outcome) (D) were deparaffinized, stained using anti-pSTAT5B, and developed with 3,3'-diaminobenzidine tetrahydrochloride with an enhancer. Original magnification, $\times 100$.

expression of STAT5 (6), and the IL4 level is increased by STAT5 signaling (7), we next employed IHC staining of POSTN and IL4 in the samples of lesional skin of patients with MCC. In the stromal areas of MCC, POSTN deposition was positive (Figure 2A and B) in 23 cases (96%). Only one case (4%) who died of MCC was negative for POSTN (Figure 2C). In contrast, the expression of IL4 was detected in 50% (8/16) of MCC cases with a good outcome, and 50% (4/8) of MCC cases with a poor outcome. The intensity of IHC staining, as scored on the semi-quantitative scale, are shown in Table I.

Sub-populations of TILs in the lesional skin of patients with MCC. Since the expression of POSTN and IL4 is prominent in the stromal area of various skin tumors, such as mycosis fungoides (8), and POSTN and IL4 both stimulate tumor-associated macrophages (TAMs) to produce chemokines and

MMPs that promote tumor development, we next employed IHC staining of CD163 and CD206 in skin samples from patients with MCC. CD163⁺ TAMs (Figure 3A) and CD206⁺ cells (Figure 3B) were detected both in MCC cases with good outcome and those with poor outcome; there was no significant difference between the numbers of CD163⁺ TAMs and CD206⁺ cells in each group (Table II).

Since Paulson *et al.* reported an association between intratumoral CD8⁺ lymphocyte infiltration and MCC-specific survival (2), we employed IHC staining of CD8⁺ cells, granzysin-bearing cells, and FOXP3⁺ regulatory T-cells (Tregs) in skin samples from patients with MCC. CD8⁺ cells, granzysin-bearing cells⁺ (Figure 3C), and FOXP3⁺ Tregs (Figure 3D) were detected both in MCC cases with a good and those with poor outcome. As a previous report suggested (2), the number of CD8⁺ cells was significantly increased in patients with a good outcome ($p=0.04$), whereas no significant

Table II. Summary of the average number of immunoreactive cells in Merkel cell carcinoma.

Marker	Good outcome (cell number/HPF)	Poor outcome (cell number/HPF)	p-Value
CD8	362.5	148	0.04
Granulysin	79.7	166.3	0.51
CD163	111.3	119.3	0.86
CD206	82.3	130	0.15
FOXP3	14.5	13	0.86

HPF: High-power field; FOXP3; forkhead box P3; FOXP3.

difference in the numbers of granulysin-bearing cells and FOXP3⁺ Tregs was seen between the groups (Table II).

MMP12 expression in the lesional skin of patients with MCC. Since previous reports also suggested that POSTN and IL4 stimulate CD163⁺ macrophages to increase *MMP12* mRNA expression (8, 9), we employed IHC staining of *MMP12* in skin samples from patients with MCC. *MMP12* has been shown to correlate with a poor prognosis in various types of skin cancer (10, 11). Deposition of *MMP12* in the stromal area was detected both in MCC cases with good and those with poor outcome, and there was no significant difference between the intensity of IHC staining in each group (Table I). Notably, in two cases of MCC with spontaneous regression, no deposition of *MMP12* was detected. Representative images are shown in Figure 4.

Discussion

Recent reports have suggested that the nuclear localization of STAT5 might be a prognostic marker for several cancer types (12). For example, Barash *et al.* reported that STAT5 is a marker of a good outcome in patients with estrogen receptor/progesterone receptor-positive breast tumors (5). In contrast, STAT5 phosphorylation has been reported to be associated with a poor prognosis in urothelial carcinoma and prostate cancer (13, 14). Moreover, the inhibition of Janus kinase 2 (JAK2)-STAT5 signaling even suppressed tumor growth in primary prostate cancer (14). Since these reports did not distinguish between the isoforms of STAT5 (STAT5A and STAT5B), this discrepancy might have been caused by differences in the expression of these STAT5 isoforms. Notably, O'Shea *et al.* reviewed JAK and STAT signaling in cancer, and reported that STAT5A activation is associated with cancer growth, whereas STAT5B deficiency results in immunodeficiency (12). As Nghiem *et al.* suggested from a clinical trial of pembrolizumab for MCC, immunotherapy [e.g., antibody to programmed cell death 1 (PD1)] is one of the most

Figure 2. Periostin expression in the lesional skin of patients with Merkel cell carcinoma (MCC). Representative images for tissues scored as intense (++) (A), moderate (+) (B), and negative (-) (C) staining are shown. Sections were deparaffinized and stained using anti-periostin. The sections were developed with liquid permanent red. Original magnification, $\times 100$.

Figure 3. CD163⁺ tumor-associated macrophages, CD206⁺ cells, granulysin-bearing cells, and forkhead box P3 (FOXP3)⁺ regulatory T cells in the lesional skin of patients with MCC. Representative sections from paraffin-embedded tissue samples of the lesional skin of patients with MCC are shown. Sections were deparaffinized and stained using anti-CD163 (A), anti-CD206 (B), anti-granulysin (C), or anti-FOXP3 (D). The sections were developed with liquid permanent red. Original magnification, ×200.

promising therapies for metastatic MCC (15). Taken together, these results suggest that immunodeficiency caused by STAT5B deficiency in MCC might impair antitumor immunity in the tumor microenvironment, leading to poor outcome in MCC.

Not only tumor-derived factors, but also the cancer stroma are important for predicting prognosis of patients with cancer (10). Since the expression of POSTN regulates the expression of STAT5 (6), we evaluated the deposition of POSTN. POSTN is an extracellular matrix protein that is involved in modulating cell functions, such as chemokine/cytokine production from macrophages (16). Recently, Zhou *et al.* reported that periostin secreted from glioblastoma stem cells induces the recruitment of monocytes from peripheral blood, which results in an increase in M2 TAMs and promotes the growth of glioblastoma multiforme (17). In addition, as we previously reported, POSTN stimulates TAMs to produce proinflammatory chemokines and MMP12, which are involved in the formation of cutaneous T-cell lymphomas (8, 10, 18). These reports further suggested that

periostin in the tumor microenvironment plays roles in the recruitment of M2 TAM precursors and in M2 polarization of macrophages in the tumor microenvironment to promote tumor development. In the present study, all cases, except for one with poor outcome, expressed POSTN, and there was no significant difference in the number of TAMs and the expression of MMP12 in each group, suggesting that the POSTN/TAMs/MMP12 axis is not related to the prognosis of patients with MCC.

Since Paulson *et al.* reported an association between intratumoral infiltration of CD8⁺ lymphocytes and MCC-specific survival (2), and as Nghiem *et al.* suggested from their clinical trial of pembrolizumab for metastatic MCC (15), it is important to investigate the subpopulations of TILs for predicting the prognosis of MCC. Therefore, we employed immunohistochemical staining of CD8, granulysin, and FOXP3. Granulysin has homology with other cytotoxic molecules of the saponin-like protein family (19), and it lyses various tumors, which is how it might be

Figure 4. The deposition of matrix metalloproteinase 12 (MMP12) in the lesional skin of patients with Merkel cell carcinoma (MCC). Representative images for intense (++) (A), moderate (+) (B), and negative (-) (C) staining are shown. Paraffin-embedded tissue samples of the lesional skin of patients with MCC were deparaffinized and stained using anti-MMP12. The sections were developed with liquid permanent red. Original magnification, $\times 100$.

related to the prognosis of patients with cancer (19-21). FOXP3⁺ Tregs play a pivotal role in maintaining peripheral tolerance that actively suppresses effector T-cells (22). At the tumor site, in concert with TAMs, Tregs maintain the immunosuppressive microenvironment and promote tumor growth (23); as such, FOXP3 could therefore be a target for immunotherapy (24). In the present study, unlike for CD8⁺ cells described in a previous report (2), there was no significant difference in the numbers of granulysin-bearing cells and FOXP3⁺ Tregs in each group.

In this report, we described 16 cases of MCC with a good outcome, all of which expressing pSTAT5B, and eight cases of MCC with a poor outcome, none of which expressing pSTAT5B. Moreover, we additionally employed IHC staining of POSTN and IL4, as well as for subpopulations of TILs (granulysin-bearing cells, Tregs, CD163⁺ cells, and CD206⁺ cells), and the deposition of MMP12 in the lesional skin of patients with MCC. The results suggest that there is no significant difference in stromal factors between MCC cases with a good outcome and those with a poor one. Our study demonstrates that pSTAT5B could be a marker for improved outcome in MCC.

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None.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

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