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ORIGINAL ARTICLE

A clinicopathological analysis of primary mucosal malignant melanoma

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Abstract
Purposes Primary mucosal malignant melanoma (PMMM) is a rare and highly lethal neoplasm associated with a poor prognosis. CXC chemokine receptor 4 (CXCR4) is expressed on various tumor cells, including malignant melanoma. Recent data indicate that CXCL12 and CXCR4 play a critical role in the behavior of cancer cells and in the survival of cancer patients. However, there has been no study that has addressed the expression and function of CXCR4/CXCL12 signaling in PMMM.
Methods Immunohistochemical staining for CXCL12 and Ki67 in biopsy tissues from 10 cases of PMMM was performed. We analyzed the correlations between the clinicopathological features and expression levels of CXCL12 and Ki67.
Results Six cases showed a high level of CXCL12 expression, while four cases had a low level of expression. High expression of CXCL12 correlated with a poor prognosis, although statistical significance was not reached ($p = 0.054$). Ki67 was highly expressed in five cases, while the expression in the other five cases was low. There was no correlation between the Ki67 expression and prognosis.
Conclusions The findings of this study suggest that CXCL12 expression may play an important role in the biological behavior of PMMM and may be associated with a poor prognosis of PMMM patients.

Keywords Primary mucosal malignant melanoma · CXCL12 · CXCR4

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Purposes

Melanoma is one of the most aggressive human cancers once metastasis begins. Therefore, it is important to characterize the molecular factors involved in melanoma dissemination. Melanomas are malignancies that can affect any anatomical region where melanocytes exist (epidermis, eyes, nasal cavity, oropharynx, rectum and anus). The most common form of melanoma involves the epidermis, and constitutes 91.2 % of melanoma cases, whereas mucosal melanoma accounts for 1 % of cases [1]. Mucosal melanomas arise in the nasal cavity, oral cavity, esophagus, anorectal regions and other sites. Primary esophageal malignant melanoma (PEMM) is a rare neoplasm, and accounts for only 0.1–0.2 % of all esophageal malignant tumors [2, 3]. Only 0.5 % of all noncutaneous melanomas are found in the esophagus [4]. PEMM is characterized by aggressive local invasion and multiple and early metastasis. Despite the availability of various therapies, such as radical resection, chemotherapy, radiotherapy, immunotherapy and hyperthermia, the prognosis of PEMM patients is still poor. Since PEMM was first reported in 1906, approximately 300 cases have been reported in the literature [5]. In two large retrospective studies, the median survival was 10–13 months, and the 5-year survival was less than 5 % [6].

Primary anorectal malignant melanoma (PAMM) is also a rare and highly lethal neoplasm associated with a poor

prognosis, which was first reported in 1857. Anorectal melanomas account for fewer than 25 % of all mucosal melanomas. PAMM accounts for only 0.8 % of anorectal malignancies. Because 61 % of patients with PAMM already have distant metastases at the time of diagnosis, the prognosis is very poor, with a median post-treatment survival time of 12–20 months and a 5-year survival rate of 6–22 % [1].

Chemokines are part of a superfamily of small structurally related chemoattractant cytokines. They bind to G-protein-coupled receptors on leukocytes and stem cells, and work through guanine-nucleotide-binding (G) proteins to initiate intracellular signaling cascades that promote migration towards the chemokine source. Chemokines are classified into four groups (CXC, CX3C, CC and C) according to their amino acid sequences. CXC chemokine receptor 4 (CXCR4) is expressed on tumor cells, such as primary malignant cutaneous melanoma, breast, prostate, colon, pancreatic and ovarian cancers [7–9]. A recent study showed that the assessment of CXCR4 immunoexpression was a novel tool for predicting the tumor aggressiveness in malignant cutaneous melanoma, and in particular, that a high immunoexpression level of CXCR4 and CXCL12 in tumor cells might be a sign of a poor prognosis [10]. Previous reports also described that the MIB-1 immunoreactivity correlates with the metastatic dissemination and prognosis of primary cutaneous malignant melanoma [11].

However, there have been no studies that have addressed the expression and function of CXCR4/CXCL12 signaling in primary mucosal malignant melanomas (PMMMs). In this study, we immunohistochemically analyzed biopsied and resected tissues, which were formalin-fixed, paraffin-embedded PMMMs, to investigate the utility of assaying the immunoexpression of CXCL12, and to determine the correlation between the clinical features and CXCL12 expression.

Methods

Patients

Ten cases of PMMM, including seven PEMMs and three PAMMs that were referred to Kumamoto University Hospital during an 11-year period from 2001 to 2011, were identified in the medical records. All ten cases of PMMM were reported based on the clinicopathological correlation, with no history of melanoma. Each patient's clinical information, including the age, gender, clinical presentation, treatment and pathological findings, was obtained from our medical record database. The overall survival was calculated from the time of diagnosis of PMMM to the time of the last follow-up.

Immunohistochemical staining

An immunohistochemical analysis of the expression of HMB-45, melan A, S-100 and pan-cytokeratins was performed in all cases to confirm the diagnosis of malignant melanoma. In addition, an immunohistochemical analysis of CXL12 and Ki67 was conducted on the same cases. The antibodies and dilutions used were HMB-45 (1:50; Dako, Carpinteria, CA, USA), melan A (1:50; Dako, Carpinteria, CA, USA), S-100 (1:2; Nichirei Biosciences Inc., Tokyo, Japan), pan-cytokeratin (1:2; Nichirei Biosciences Inc., Tokyo, Japan), CXCL12 (1:100; R&D Systems, Inc. Minneapolis, MN, USA) and Ki67 (1:100 Dako, Carpinteria, CA, USA). Standard immunohistochemical staining techniques were used as described previously [12]. Appropriate positive and negative controls were used for all studies. All IHC staining was scored independently by two pathologists. The immunohistochemical staining was scored as described below: the intensity of staining was scored as 0, 1, 2 or 3 indicating absent, weak, moderate or strong expression, respectively. The percentages of positive cells were scored as 0 for 0 %, 1 for 1–5 %, 2 for 5–25 %, 3 for 25–50 %, 4 for 50–75 %, and 5 for 75–100 %. The multiplication of these two scores was used to identify the CXCL12 expression level: low (0–8) vs. high (9–15). The results for Ki67 were categorized as high or low according to the number of positive cells. Cases were categorized as belonging to the high group if the MIB-1 index was more than 10 %. A pathologist reviewed all of the hematoxylin–eosin stained slides.

Statistical analysis

The statistical analysis was performed using JMP software program, ver.10.0.2 (2012 SAS Institute Inc.). The Mann–Whitney *U* test was used to compare continuous variables between the two groups.

Results

The clinical features of the 10 patients with PMMM are shown in Table 1. Among the seven patients with PEMM, five (71 %) were male and two (29 %) were female, while three patients with PAMM were all female. The median age of patients with PEMM was 67 years old (range 57–84), and the median age of the patients with PAMM was 71 years old (range 70–74). Although seven of the 10 patients were scheduled to undergo surgical treatment, two cases underwent only exploratory laparotomy because of the detection of distant metastasis (peritoneal dissemination and liver metastasis). Two of the seven patients with PEMM underwent esophagectomy, and the others were

Table 1 The clinicopathological features of the PMMM cases

Organ	Age (years)	Sex	Complaints	Site	Size (cm)	Type	Color	TNM	Stage	ly	v	Treat	Prognosis (days)	Cause	CXCL12	Ki67	MIB-1 (%)
Esophagus	65	M	None	Lt-Ae	6	1	Black	T2N4M1	IVb	NA	NA	Chemo-therapy	520	Alive	High	Low	5
	66	M	Dysphagia	Mt	6	2	Black	T3N2M0	III	NA	NA	Chemo-therapy	452	Dead PMMM	High	High	33
	57	M	None	Lt-Ae	1	0-llc	Normal	TlbNOMO	I	+	−	Radical resection	909	Alive	Low	Low	9
	60	F	Epigastralgia	Mt, Lt-Ae	S, 15	1	Black	T3N0M0	III	NA	NA	Chemo-therapy	60	Dead PMMM	High	High	17
	84	M	Dysphagia	MtLt	9	0-l + llb	Black	T2N2M1	IVb	NA	NA	Radiation	200	Dead PMMM	High	High	15
	68	F	Neck tumor	Ut	6	0-llc	Black	T2N4M1	IVb	NA	NA	Radical resection	7327	Dead PMMM	Low	High	19
Rectum	74	M	Dysphagia	Lt	NA	0-lsp	NA	T1bN1M0	ll	NA	NA	Radical resection	685	Dead PMMM	High	Low	1
	70	F	None	Rb	NA	1	Na	NA	Na	NA	NA	Immuno-therapy	600	Alive	High	Low	5
	71	F	None	P-Rb	2	2	Black	T1N0M0	I	−	−	Radical resection	1771	Alive	Low	High	29
	68	F	None	Rb	NA	1	Black	T3N0M0	1 Mb	+	+	Radical resection	407	Dead PMMM	Low	Low	7

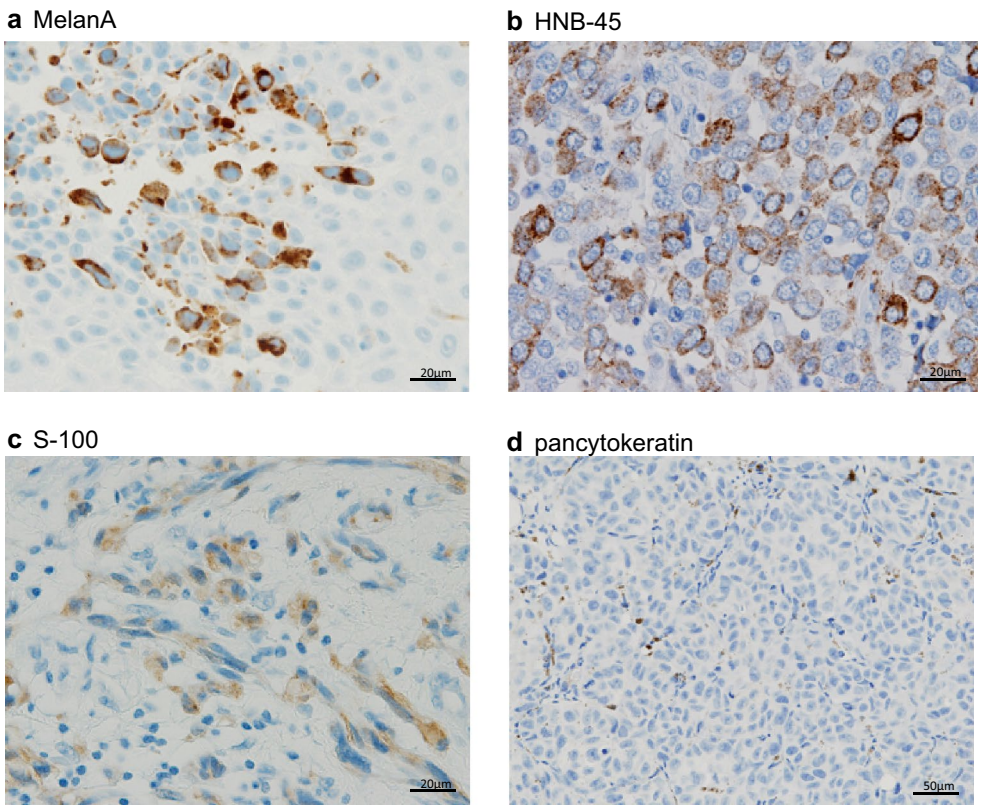

Fig. 1 Confirmation of the diagnosis for all cases by an immunohistochemical analysis of biopsy specimens. Malignant cells were positive for melan A, HNB-45 and S-100 in all cases (**a**–**c**). In contrast, they were negative for pan-cytokeratins (**a**). *Scale bars* 20 μm (**a**–**c**), 50 μm (**d**)

treated with chemotherapy (DAV-feron therapy; dacarbazine, nimustine, vincristine and interferon beta) or radiotherapy. Of the three patients with PAMM, two underwent abdominoperitoneal resection and the third was treated with immunotherapy. All five patients who underwent curative resection were treated with DAV therapy (dacarbazine, nimustine and vincristine) as adjuvant chemotherapy. Two of the five patients who underwent curative resection relapsed 1 year after surgery. One had lung, liver and mediastinal lymph node recurrence. The other had lung, liver and locoregional recurrence. Although six of the ten patients with PMMM experienced cancer-related death within 2 years from the time of their first visit to our institution, two patients survived for longer than 5 years. The recurrent sites of the deceased patients were the liver (five cases), lymph node (one case) and anastomotic site (one case).

Immunohistochemical staining

An immunohistochemical analysis of biopsies was performed for all cases to confirm the diagnosis. The malignant cells were positive for melan A, HNB-45 and S-100 in all cases (Figs. 1a, b, c). In contrast, they were all negative for pan-cytokeratins (Fig. 1d).

Representative results from the immunohistochemical staining of CXCL12 are shown in Fig. 2. The immunolabeling intensity in tumor cells varied widely. Six cases showed high-level expression of CXCL12, while in four cases, the expression levels were low. An immunohistochemical analysis of CXCL12 was performed for the resected tissues for the five cases who underwent radical resection. The results of the resected specimens were the same as those of the biopsies, and no heterogeneity was found. These results showed that there was a potential correlation between the CXCL12 expression and prognosis (Fig. 3a); however, statistical significance was not reached. We also performed immunohistochemical staining for Ki67 and determined MIB-1 index. Based on MIB-1 index, five cases were categorized into Ki67 high group, while the other five cases were categorized into Ki67 low group. There was no correlation between the Ki67 expression and the patient prognosis (Fig. 3b).

Discussion

In the current study, we have presented the clinicopathological features of 10 PMMM cases; seven PEMM cases and three PAMM cases. PMMM is an aggressive disease and shows early dissemination via the bloodstream and lymphatics. Previous studies showed that the ligands of CD44, such as osteopontin and hyaluronic acid, interacted with CD44 and induced matrix metalloproteinase-2 (MMP-2)

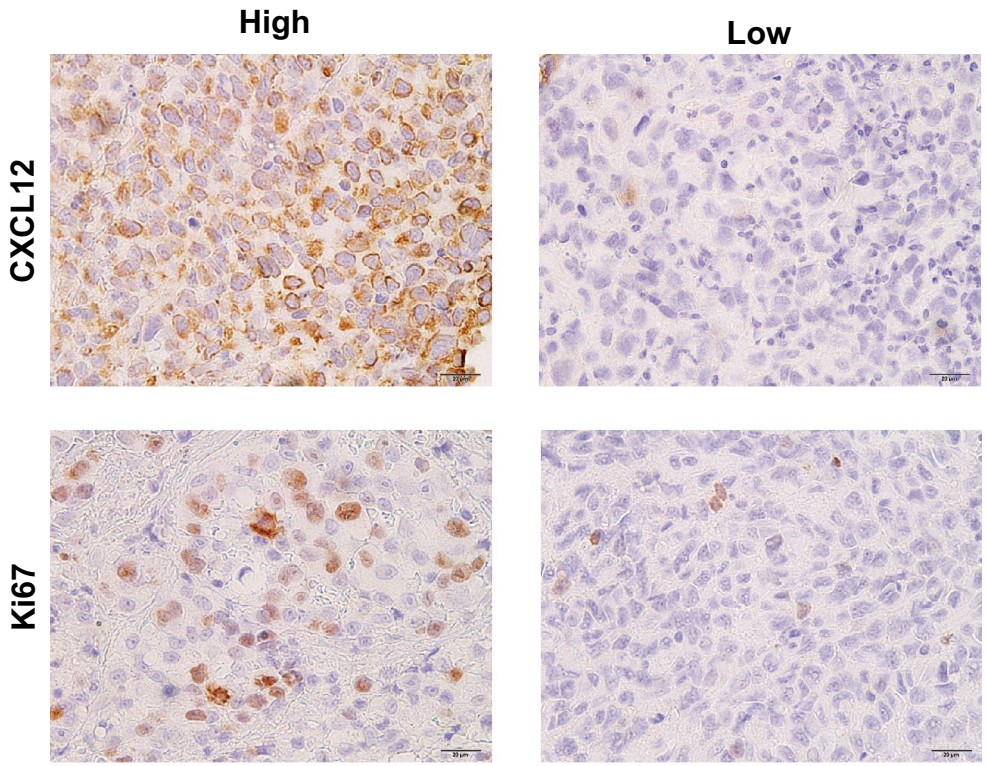

Fig. 2 The expression of CXCL12 and Ki67 in melanoma cells. Immunohistochemical staining of CXCL12 and Ki67 in melanoma cells. *Scale bar* 20 μm

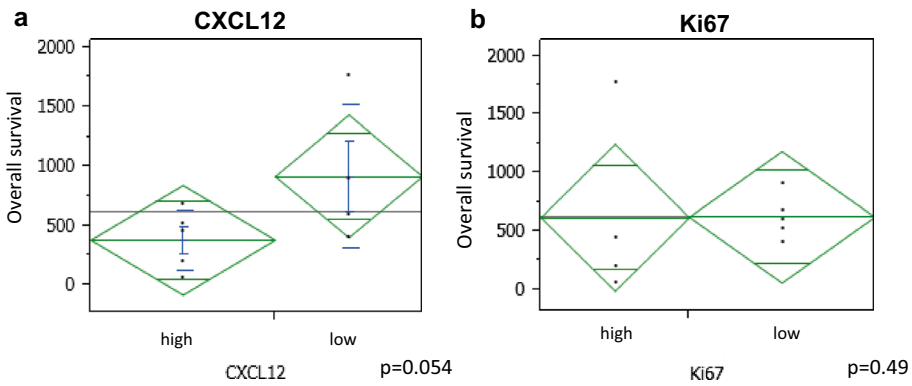

Fig. 3 The correlations between the prognosis and expression levels of CXCL12 and Ki67. **a** The results showed an apparent, although non-statistically significant correlation between the CXCL12 expression and a poor prognosis. **b** There was no correlation between the Ki67 expression and the prognosis

production, which was associated with enhanced migration and invasion of human melanoma cells [13–15]. In another report, TGF-β signaling was shown to be required for the activation of fibroblasts and their promotion of the invasive growth of melanoma cells [16]. Taken together, these studies suggest that the tumor microenvironment surrounding melanoma cells may contribute to their invasion and metastasis.

The expression of chemokine receptors by malignant cells could also represent a mechanism for increased proliferation and cell motility, thus leading to tumor growth and metastasis [10]. Many retrospective studies have now documented that the expression of various chemokine receptors is associated with a poor prognosis [17–19]. CXCL12, also called stromal cell-derived factor 1 (SDF-1), and CXCR4 play a critical role in the behavior of cancer cells and maintaining the cell migration, proliferation and survival. A recent study showed that there was a correlation between a high clinical risk and high-level expression of CXCR4 and CXCL12 in cutaneous malignant melanoma. They also described that CCR6 and CCR7 immunoexpression was also correlated with some clinical parameters, but did not seem to be more useful than CXCR4. These data suggest that the assessment of CXCR4/CXCL12

expression is a novel tool for predicting the aggressiveness of malignant melanoma. [10]. Furthermore, CXCL12/CXCR4 signaling has been reported to be an important factor associated with the invasion and metastasis of many gastrointestinal cancers, including gastric, colon and pancreatic [20, 21–22].

On the other hand, correlations between a poor prognosis and high-level growth, such as that indicated by Ki67 expression or the MIB-1 index, have also been reported. These growth indices provide independent prognostic information that can potentially be used in risk-based management of cutaneous melanoma patients [23]. In addition, many studies have reported that the MIB-1 index is important to assess the aggressiveness of various types of cancer [24, 25–26].

In this study, the immunohistochemical staining results showed that high-level expression of CXCL12 tended to correlate with a poor prognosis, whereas there was no correlation between the Ki67 expression and prognosis in PMMM in the same samples. These findings suggest that CXCL12 expression was associated with the prognosis in PMMM patients, as well as primary percutaneous malignant melanoma patients, while the Ki67 expression was not. However, the sample size of our study was too small, due to the rareness of PMMM, to conclude with statistical certainty that there was a correlation. Furthermore, there might be different biological features between PEMM and PAMM, although we analyzed them together in the present study. Therefore, further analyses with more cases will be required to verify the correlation between CXCL12 expression and the prognosis of PMMM patients, as well as to elucidate the role that CXCL12/CXCR4 signaling plays in PMMM.

Conflict of interest Daisuke Izumi, Hideo Baba and all other co-authors have no conflicts of interest to declare in association with this study.

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