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Molecular Detection and Characterization of Aneuploid CD31$^-$ Ctcs and CD31$^+$ Ctecs Expressing Epcam or Ki-67 in the Comprehensive Diagnosis of a Rare Case of Merkel Cell Carcinoma

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Abstract

Merkel cell carcinoma (MCC) is a rare, aggressive malignancy of the skin, which is an epithelial origin of cutaneous neuroendocrine cancer, with a poor prognosis. MCC frequently manifests in locations regularly exposed to the sun in older patients. Due to the absence of symbolic cutaneous symptoms, diagnosis of MCC is often overlooked at the time of presentation. The current study describes a very rare case of an 89-year-old woman, who presented with a 4 × 3 cm red nodule on the right side of her face, which was consistent with pathological diagnosis of MCC performed with histopathological and immunohistochemical examinations. In addition, the patient had a total of 35 aneuploid circulating rare cells detected, including CD31$^-$ aneuploid circulating tumor cells(CTCs) and CD31$^+$ aneuploid circulating tumor endothelial cells (CTECs), with some of them expressing EpCAM or Ki-67. Recent clinical progress with respect to MCC is thoroughly discussed, which may assist diagnosis and treatment of this unique type of carcinoma.

Keywords: Merkel Cell Carcinoma (MCC); Head and Neck Tumor; Face Tumor; Aneuploid Ctcs; Ctecs

Introduction

Merkel cell carcinoma (MCC), a rare epithelial origin of cutaneous neuroendocrine carcinoma [1] manifests most frequently on skin exposed to the sun. Toker originally referred to it as "trabecular cancer" in 1972 [2].

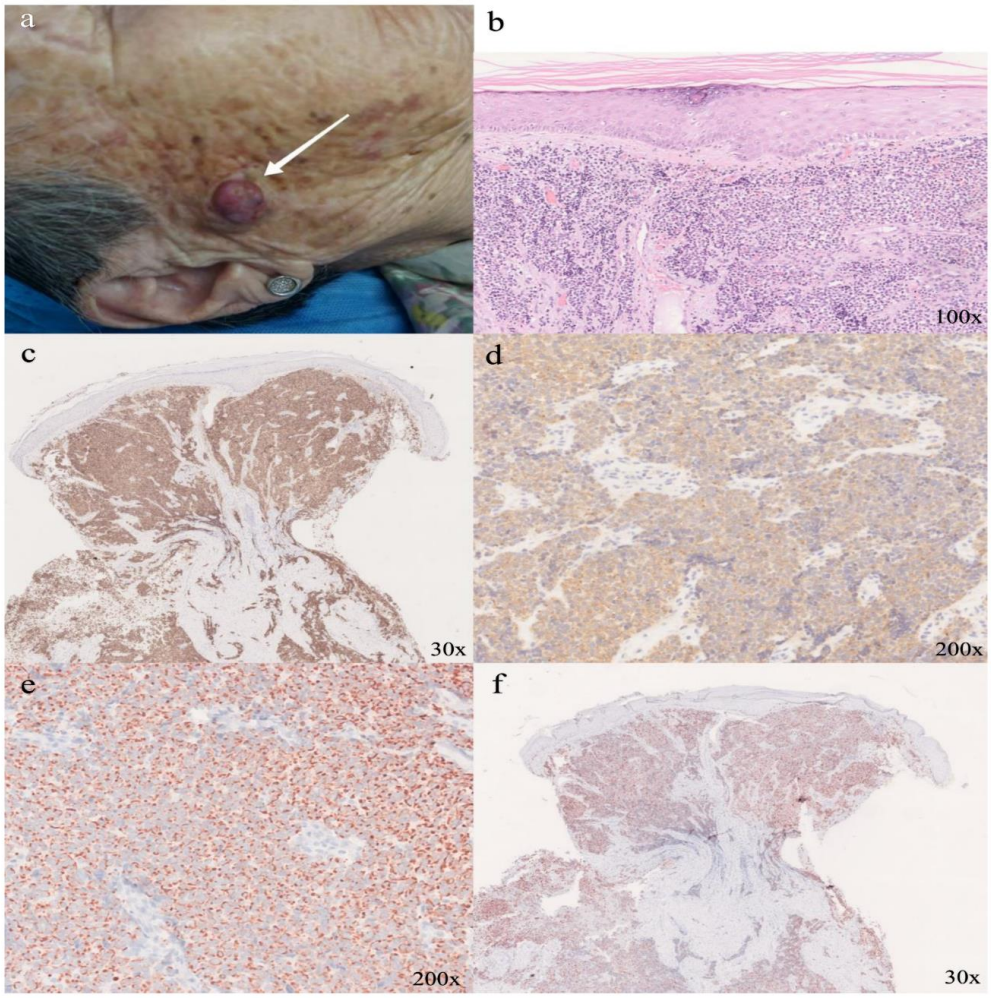
According to Olsen et al 's findings from 2021, MCC incidence increased from 1997 to 2016 at a rate of about 2-4% every year. Age-standardized incidence of MCC rose from 0.55 per 100,000 men in 1997 to 1.03 per 100,000 men in 2016, and from 0.28 to 0.45 per 100,000 women in the United States, [3] but in Brazil, the equivalent increases from 2000 to 2015 were from 0.31 to 1.21 for men and from 0.50 to 0.55 for women [4]. Additionally, men typically have a higher incidence of MCC than women.

MCC is quite common in older people and skin regions frequently exposed to the sun, such as the head and neck. The analyses of Kieran et al. show that the median age of diagnosis in Vitoria, Australia, between 1986 and 2016 was 79.5 years old [5]. The results by Olsen et al. further reveal that the age-specific incidence was highest for individuals who were 80 years old, that the increase in incidence over time was mostly confined to this age group, and that MCC of the head and neck experienced the largest increase in absolute numbers. Merkel cell carcinoma is manifested by asymptomatic erythematous/violaceous nodules or plaques, which grow rapidly and solitary. The clinical characteristics of MCC have been encapsulated by the abbreviation AEIOU, where AE stands for asymptomatic, E for quickly expanding, I for immune suppression, O for older than 50 years, and U for UV-exposed [6,7]. Here, a comprehensive cellular and molecular diagnosis of a rare case of Merkel cell carcinoma is reported. Recent progress regarding clinical manifestations, pathological features, and treatment of MCC is also discussed.

Case report

An 89-year-old woman presented with a 4-month history of a violaceous painless nodule on the right side of her face, gradually increasing. The nodule was well-circumscribed, hemispherical in shape, violaceous in color, and measured 4 × 3 cm. The mobility was acceptable, and there was no erosion, ulcer, exudation, scale, or tenderness (Fig.1A-a). Pathological diagnosis revealed that nuclear mitosis was evident, the epiderm was absent, and little round cell clusters may be detected in the deep dermis (Figure.1A-b). As shown in Figure. 1A-c, d, and e, abundant expression of Syn, CgA, and Ki-67 in the specimen was observed. In addition, immunohistochemical staining of CK20 demonstrated paranuclear dot-like staining (Figure. 1A-f). Results of additional comprehensive pathological immunohistochemistry staining are summarized in **Figure. 1B,** indicating pan-CK^{+}, Syn^{+}, CgA^{+}, P63+, EMA^{+}, CK7^{-}, SOX10^{-}, S100^{-}, Melan-A^{-}, and focal staining of CD3, CD20, CD8, CD4, and CD79a in lymphocytes. Upon histological and immunohistochemical analysis, the tumor was consistent with the diagnosis of MCC.

A Pathological diagnosis



B Summary of immunohistochemistry staining

Ki-67	pan-CK	Syn	CgA	P63	EMA	CK7	SOX10	S100	Melan-A
70%	+	+	+	+	+	-	-	-	-

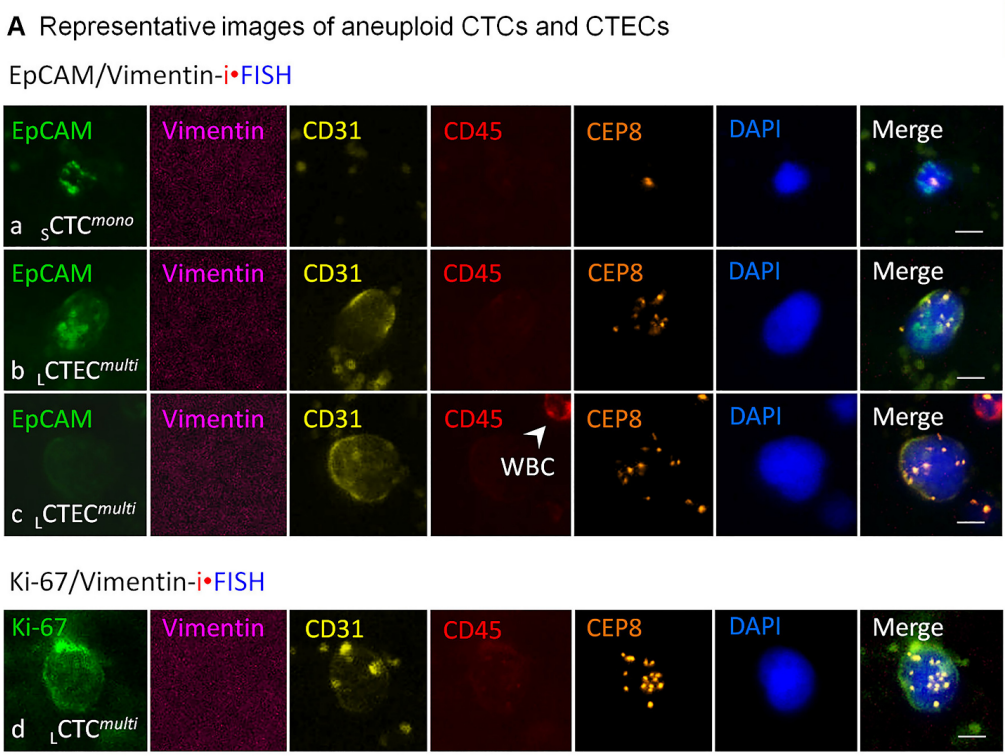
CK20	CD3	CD4	CD8	CD20	CD79a
paranuclear dot-like staining	focally stained in lymphocytes				

Figure 1: Comprehensive diagnosis of MCC. (A) Pathological diagnosis. A-a, pre-clinical pathology biopsy shows an isolated violaceous-colored nodule, about 3 x 4 cm in size on the right side of the patient's face (white arrow). A-b, H&E staining shows evident nuclear mitosis, and the little round cell clusters in the deep dermis. Pathological immunohistochemistry reveals that positive for CgA (A-c) as well as Syn (A-d), and dot-like positive staining for CK20 (A-e), about 70% positive expression of Ki-67 in the specimen (A-f). (B) Summary of comprehensive immunohistochemistry examination. Positive staining is indicated as the red +.

Aneuploid CD31- circulating tumor cells (CTCs) and CD31+ circulating tumor endothelial cells (CTECs) in this MCC patient were detected by the subtraction enrichment integrated with immunostaining-fluorescence in situ hybridization (SE-iFISH) [8]. Briefly, six ml of peripheral blood was collected from the subject, followed by subtraction enrichment processing. Enriched circulating rare cells were split, and each half specimen was subjected to 6-channel EpCAM/Vimentin-iFISH and Ki-67/Vimentin-iFISH, respectively. As illustrated in Figure 2, 25 CTCs and 10 CTECs were detected in a total of six ml of blood, including three EpCAM+ CTCs, one Ki67+ CTC, and one EpCAM+ CTEC. Most of the detected aneuploid CTCs and CTECs were EpCAM-/Ki-67- null cells. Among 25 CD31- CTCs, 60% of them (15 out of 25) were multiploid (≥ pentasomy 8), and 80% of the detected CTCs (20 out of 25) were large cells (>5 mm). Similarly, 70% of CD31+ CTECs were large cells (7 out of 10) with 80% of the detected CTECs exhibiting

multiploid Chr8 (8 out of 10). Expression of Vimentin on CTCs or CTECs was not observed in this MCC patient.

A Representative images of aneuploid CTCs and CTECs



B Quantitative and molecular characterization of aneuploid CTCs and CTECs

Classification	Cell size	Tumor markers	Ploidy: Haploid	Near-diploid	Tri-ploid	Tetra-ploid	Multi-ploid	Sum2 (size)	% (Sum2/Tot)	Total	Sum: Ep+	Ki67+	Null
CD31⁻CTC	Large	EpCAM+	0	0	0	0	0						
		EpCAM-	0	0	1	2	7	20 (large)	80% (large)				
		Ki67+	0	0	0	0	1						
		Ki67-	0	0	1	1	7						
	Small	EpCAM+	1	2	0	0	0			25	3	1	21
		EpCAM-	0	0	0	1	0	5 (small)	20% (small)				
		Ki67+	0	0	0		0						
		Ki67-	0	0	0	1	0						
		Sum1	1	2	2	5	15						
		% (Sum1/Tot)	4%	8%	8%	20%	60%				12%	4%	84%
CD31⁺CTEC	Large	EpCAM+	0	0	0	0	1						
		EpCAM-	0	0	0	0	4	7 (large)	70% (large)				
		Ki67+	0	0	0	0	0						
		Ki67-	0	0	0	0	2						
	Small	EpCAM+	0	0	0	0	0			10	1	0	9
		EpCAM-	0	0	0	1	0	3 (small)	30% (small)				
		Ki67+	0	0	0	0	0						
		Ki67-	0	0	1	0	1						
		Sum1	0	0	1	1	8						
		% (Sum1/Tot)	0	0	10%	10%	80%				10%	0	90%

Figure 2: Detection and molecular characterization of diverse subtypes of CD31⁻ CTCs and CD31⁺ CTECs. **(A)** Representative images of non-hematologic CTCs and CTECs respectively detected by EpCAM/Vimentin-iFISH and Ki-67/Vimentin-iFISH. A-a, an EpCAM⁺/Vimentin⁻/CD31⁻ haploid CTC in small cell size (≤ 5 mm, SCTC*mono*). A-b, an EpCAM⁺/Vimentin⁻/CD31⁺ multiploid CTEC in large cell size (> 5 mm, greater than or equal topentasomy 8, LCTEC*multi*). A-c, a large EpCAM⁻/Vimentin⁻/CD31⁺ multiploid null CTEC (LCTEC*multi*). A CD45⁺ white blood cell (WBC) is indicated by the white arrow. A-d, a large Ki-67⁺/Vimentin⁻/CD31⁻ multiploid CTC (LCTC*multi*). Bars, 5 mm. **(B)** Quantitative and molecular analyses. Among 25 detected CTCs, 20 of them are large cells (LCTCs, 20/25=80%), and the others are small cell sizes (SCTCs). Degrees of ploidy in CTCs are monosomy 8 (1/25=4%), near-disomy 8 (2/25=8%), trisomy 8 (2/25=8%), tetrasomy 8 (5/25=20%), and multisomy 8 (15/25=60%), respectively. Three CTCs are EpCAM⁺ (3/25=12%), and 1

CTC is Ki-67$^+$ (1/25=4%), the remaining (21/25=84%) are null cells without expression of either EpCAM or Ki-67. With respect to 10 CTECs, 7 are large cells (70%), and the rest of the CTECs are small cells (30%). Ploidies in CTECs are trisomy 8 (1/10=10%), tetrasomy 8 (1/10=10%), and multisomy 8 (8/10=80%). One CTEC exhibits a positive expression of EpCAM (1/10=10%), others are null cells.

The family of the patient stopped seeking treatment after being informed of her condition and understanding the basics of MCC. The patient is currently still alive and not in any specific discomfort.

Discussion and conclusion

Merkel cell carcinoma (MCC) is a rare and aggressive malignant tumor of the skin, which is a kind of epithelial neuroendocrine carcinoma of the skin. Its clinical manifestation is nonspecific and its prognosis is poor.

The mechanism of carcinogenesis in MCC may be related to Merkel cell polyomavirus (MCPyV) infection and UV radiation. Feng et al. showed that MCPyV infection may have a role in the pathogenesis of MCC since they found MCV sequences in 80% of MCC tumors and viral DNA clonally integrated into the genome of MCC cells in 75% of MCPyV$^+$ tumors. The preservation of the oncogenic phenotype is significantly associated with the expression of LT, which causes a certain DNA damage response.ST is more important in tumor transformation and induces tumor formation [9]. ST plays a bigger role in the transformation of tumors since it promotes tumor development, impairment, proliferation, and apoptosis and activates the DNA damage response. However, MCPyV is widespread in the population, with approximately 60-80% of the population infected with this virus. Therefore, despite the strong association of MCPyV with MCC, the exact mechanism of its carcinogenesis remains unclear [10]. A high correlation between MCC and UV radiation has been reported in the literature [11]. UV radiation can induce mutations in the viral genome, leading to DNA damage, the effects of which are more pronounced in MCPyV$^-$ tumors. In MCPyV$^-$ tumors, cells cannot effectively repair the damage caused by UV radiation and proliferate as mutations accumulate [12]. In contrast, in MCPyV$^+$ tumors, UV radiation causes tumor development by inducing immunosuppression: UV radiation induces altered expression and function of inflammatory mediators in antigen-presenting dendritic cells, which modulates immune sensitivity and causes immunosuppression. MCC is commonly seen in patients with leukemia, lymphoma, HIV infection, and immunosuppression due to organ transplantation [13]. The pathogenic mechanism of MCC due to UV radiation may explain this phenomenon: MCC patients often have other skin tumors associated with sun exposure, such as basal cell carcinoma, squamous cell carcinoma, and melanoma.

The tumor typically presents as a rapidly growing flesh-colored or violaceous nodule or plaque. MCC frequently occurs in sun-damaged skin. The most commonly affected sites, in order of frequency of involvement, are the head, neck, extremities, and trunk [14]. Because of the lack of specificity of cutaneous presentation, clinical features rarely assist with diagnosis, therefore histopathology and immunohistochemistry play a crucial role in the diagnosis.

Hematoxylin and eosin stains of MCC demonstrate an infiltrate of small round blue cells, arranged mainly in sheets and nests. And they exhibit a high nuclear: cytoplasmic ratio with a “salt and pepper” nuclear chromatin pattern and indistinct nucleoli [15,16] MCC can be differentiated from other diseases by pathological manifestations. In contrast to basal cell carcinoma, MCC lacks peripheral palisading. In contrast to melanoma, MCC usually spares the epidermis and is free of pigment [17] MCC cells are positive for several types I or type II cytoskeletal keratins, particularly CK20 with a distinctive perinuclear dot staining pattern. The tumor also expresses neurofilament (NF) and neuroendocrine markers such as chromogranin A and synaptophysin. The co-expression of cytokeratins and neurofilament is characteristic of MCC. Recently, insulinoma-associated protein 1 (INSM1) is a sensitive nuclear marker of MCC [13,17].

MCC has a poor prognosis, a low survival rate, and is highly aggressive. Harms et al. analyzed data related to 9387 cases of MCC and came to the following conclusions: Five-year OS estimates for c patients with localized MCC were 51%, while with the nodal disease was 35%, and for patients presenting with distant metastatic disease was 14% [18]. The analysis by Maloney et al. showed that 42.3% of cases with a head/neck primary site of MCC had liver

metastases, and 39.6% of cases with a trunk primary site of MCC had bone metastases. Increasing age, and liver and brain metastases were independent prognosticators of poorer prognosis [19].

In this case, it is the first time SE-iFISH was employed to detect the CTCs and CTECs in the MCC patient. Circulating tumor cells (CTCs) are considered the real-time liquid biopsy for patients with cancer [20]. The majority of endothelial cells in tumor vasculatures are tumor-derived endothelial cells (TECs). TECs contribute to morphological abnormal tumor vasculature, leading to increased vascular permeability and transendothelial intravasation as well as extravasation during tumor metastasis. CTECs are TECs shed into the peripheral blood [21]. CTCs and CTECs constitute a pair of circulating tumor biomarkers in cancer patients [22]. Detection and molecular characterization of CTCs and CTECs may offer real-time insights into the course, prognosis, and effectiveness of cancer treatment [20].

The occurrence, development, and outcome of human diseases could be considered a spatiotemporal ecological process [23]. The tumor microenvironment, which is a complex, dynamic entity, has been proven to actively assist the process of tumor development [24]. The direct interaction of CTCs with TMEs is essential for tissue invasion and tumor metastasis. The association between neutrophils and CTCs promotes cell cycle development in circulation and expands the metastatic potential [25]. Also, the association between CTCs and tumor-associated macrophages (TAMs) promotes advanced tumor metastasis. Chen et al. found that the JAK2/ STAT3/ miR-506-3p/ FoxQ1 axis is regulated by TAMs to enhance colorectal cancer migration, invasion, and CTC-mediated metastasis [26]. According to the research, a significant number of CTCs are detected in many solid metastatic tumors, and several malignancies of epithelial origin, including pancreatic, colorectal, and hepatocellular carcinomas, also exhibit a significant number of $EpCAM^+$ CTC.[25] As the study by Allen et al. shows, 6 of 11 colorectal cancer patients with liver metastases had positively significant numbers of apoptotic CTCs in peripheral blood as measured by Fischer's exact test [27]. So it is clear that CTCs have the potential to cause distant cancer metastases and that CTCs can be used as a dynamic tool to assess the metastatic tendency of tumors and to evaluate prognosis.

SE-iFISH enables to effectively enrich non-hematologic circulating rare cells regardless of cell sizes and cell surface molecule (such as EpCAM) expression, followed by *in situ* karyotypic and phenotypic co-examination of chromosomal aneuploidy as well as protein expression of various tumor markers [8,22]

The epithelial molecule EpCAM participates in epithelial-to-mesenchymal transition (EMT) and cancer metastasis [28,29]. As previously reported, the detection of $EpCAM^+$ aneuploid CTCs could be utilized to evaluate surgical efficacy and to predict poor prognosis and tumor recurrence in malignancies, such as hepatocellular carcinoma and breast cancer [30,31] Besides, abundant expression of Ki-67 was reported to be highly associated with cancer cell proliferation, growth, metastasis, and the tumor's clinical stage [32,33]. Moreover, the degree of aneuploidy is relevant to tumor grades as well as cancer cell proliferation, showing the higher degree of chromosomal ploidy, the higher grade of the tumor [34]. In regards to MCC CTC, Boyer et al.'s research showed that CTCs were detected mainly in patients with stage III/IV MCC, but also in some patients with early-stage MCC [20]. Riethdorf et al.'s research showed that up to 55% of patients with MCC had one or more CTCs/7.5 ml detected, and among those MCC patients, CTCs were detected in 70% of subjects with lymph node metastases and in all patients with distant metastases [35].

In the current presented case, 25 $CD31^-$ CTCs and 10 $CD31^+$ CTECs were detected, with some of them expressing EpCAM or Ki-67. The majority of detected CTCs and CTECs were large multiploid cells. Obtained results suggested that the patient might be undergoing an active distant tumor metastasis and might have a poor prognosis. Longitudinal monitoring of $CD31^-$ CTCs and $CD31^+$ CTECs along the following treatment will provide a feasible and applicable tumor liquid biopsy approach which enables a more effective assessment of subsequent therapeutic efficacy in this patient, ultimately illustrating the clinical significance of the detected MCC CTCs and CTECs, particularly those expressing EpCAM or Ki-67.

The first-line treatment for patients with primary Merkel cell cancer is surgery. Advocate adjuvant radiotherapy (RT) after full excision with clinical safety margins of 1 cm as the optimal course of action [36]. Mohs

micrographic surgery (MMS) or wide local excision (WLE) is the latest surgical excision technique [15]. We assessed the results of primary MCC patients receiving MMS as monotherapy at a single institution, there was no local recurrence. Stage I malignancies had a 5-year MCC-specific survival rate of 91.2% (historical controls ranged from 81% to 87%). Stage IIA cancers had a 68.6% 5-year MCC-specific survival rate (historical controls ranged from 63% to 67%). A survival rate that is at least as excellent as WLE+RT may be available with Mohs surgery alone [37].

The 5-year local relapse-free rate following treatment with radiotherapy alone is as high as 90% in MCC, a highly radiosensitive tumor. Radiation therapy is therefore an effective alternative therapy for patients with advanced MCC, MCC patients with primary lesions that are difficult to perform surgery on, and inoperable patients [38]. Sentinel lymph node biopsy can clinically identify lymph node metastases and indicate lymph node dissection. According to the study by Harounian et al., 29% of the clinically N0 MCC patient group who underwent SLN examination had pathologic proof of metastases. The existence of involvement changed the tumor stage and the size of the adjuvant radiation field in comparison to tumor-free SLN [39].

Nowadays, Anti-PD-1/PD-L1 agents have achieved significant efficacy in treating metastatic MCC (mMCC). Avelumab, an anti–PD-L1 monoclonal antibody, became the first treatment for patients with mMCC to receive approval in the United States, the European Union, and Japan. An analysis of a clinical trial showed that avelumab had antitumor activity and a manageable safety profile in patients with mMCC [40]. Nivolumab can mediate substantial tumor regression. Data from Topalian et al. showed that 47.2% of patients treated with Nivolumab for 4 weeks followed by surgery achieved a pathologic complete response (pCR, which was defined as the absence of residual viable invasive cancer on completely resected tumor specimens including all sampled lymph nodes). 87.9% of patients show radiographic tumor reduction [41].

Ethics statement

The study was conducted according to the Declaration of Helsinki Principles. An informed consent form, approved by the Ethics Review Committees (ERC) of the Dermatology Hospital of Southern Medical University, Guangzhou, China, was signed and obtained from the patient.

Author contributions

SRL, SUL, NW, JX, JR, and RC participated in patient treatment and analysis of results. SRL and AYL contributed to writing the original draft. AYL and DDW contributed methodology and validation. RC and PPL contributed to the conceptualization, reviewing, and editing of the manuscript. All authors approved the submitted version of the manuscript.

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Conflict of Interest

i•FISH® is the registered trademarks of Cytelligen. PL is the president at Cytelligen. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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