Archives of Dermatological Research (2019) 311:37–43
https://doi.org/10.1007/s00403-018-1877-x

ORIGINAL PAPER

Evidence of proliferative activity in human Merkel cells: implications in the histogenesis of Merkel cell carcinoma

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Received: 21 June 2018 / Revised: 25 October 2018 / Accepted: 11 November 2018 / Published online: 20 November 2018

Abstract

The cellular origin of Merkel cell carcinoma (MCC) is controversial. We previously hypothesized that MCC originates from hair follicle stem cells or Merkel cell (MC) progenitors residing within the hair follicle bulge. Examination of three cases of combined MCC led to the unexpected discovery that numerous keratin 20 (CK20)-positive MCs within the squamous cell carcinoma (SCC) component of combined MCC appeared morphologically normal with dendritic and oval shapes. Moreover, one extremely rare case of combined SCC and MCC showed both intra-epidermal and dermal MCCs. These three cases represent the first documentation of MC hyperplasia in MCC, besides various benign follicular neoplasms associated with MC hyperplasia. Therefore, to elucidate the proliferating potential of MCs and their histogenetic relationship with MCCs, we further investigated these cases based on pathological observations. We identified numerous cells co-expressing CK20 and the proliferation marker Ki-67, identical to the morphological and immunohistochemical features of normal MCs. This finding indicated that MCs can no longer be considered as pure post-mitotic cells. Instead, they have proliferative potential under specific conditions in the diseased or wounded skin, or adjacent to various skin tumors, including MCC. Intimate co-existence of two malignant cell components composed of intradermal and intra-epidermal MCCs, with the proliferation of normal-appearing MCs in the same lesion, lends support to the hypothesis that MCs and MCC cells are derived from MC progenitors residing within the hair follicle bulge. Specifically, MCCs are derived from transformed MC progenitors with potential for dual-directional differentiation towards neuroendocrine and epithelial lineages.

Keywords Merkel cell carcinoma · Hair follicle · Progenitor cells · Proliferation · Squamous cell carcinoma · Histogenesis

Introduction

To date, the cellular origin of Merkel cell carcinoma (MCC) remains controversial. Human hair follicles (HFs) harbor both epithelial HF stem cells (eHFSCs) and progenitor cells (PC) [3, 5, 6, 10, 12, 14, 20, 29–32, 37]. In addition, the outer root sheath, which is present in the well-defined hair follicle bulge (HFB) at the arrector pili muscle attachment site, contains various PC population that differentially express eHFSC-associated markers [3, 5, 6, 12, 14, 27, 29–33, 37]. Since HFB cells play an important role during skin tumorigenesis [5], we particularly focused on the HFSCs that reside within the HFB to investigate the histogenetic association between Merkel cell (MC) and MCC cells.

Whether MCs arise from epidermal or neural crest PCs has long been inconclusive. In mice, MCs arise from stem cells (SCs) or PCs of the epidermal lineage [23, 39, 40]. In the human skin, a large proportion of MCs is localized in the HF and touch dome, whereas MCs are sparsely present in the inter-follicular epidermis. Moreover, MCs are preferentially located within the HFB area, not only in the terminal HFs of the scalp skin but also in the vellus HFs that cover most of the body surface [25, 26].

Although it is generally considered that HFSCs reside in the bulbar area of HFs, accumulating evidence now indicates that keratinocyte SCs reside in the HFB area of HF in rodents as well as in humans [2, 4, 17, 22, 27, 28]. The identification of HFSC localization, as well as the preferential localization of MCs within the HFB area, led us to hypothesize that the MCs in HFs were derived from the SCs or PCs of HFB. In addition, MCC or MC hyperplasia has been observed in various benign follicular neoplasms and hamartomas, including desmoplastic trichoepithelioma,

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trichoblastomas, nevus sebaceous, fibrofolliculoma, and trichofolliculoma [7–9, 13, 16, 34–36, 41]. This characteristic feature might support our hypothesis regarding the HFSC origin of MCs.

Nevertheless, the cellular origin of MCC remains unclear. Epidermal and dermal SCs or PCs are currently regarded as the potential cells of origin for MCC [11, 18, 39]. The previous assumption of MCs being the most probable cells of origin for MCC is now largely rejected given the evidence of MCC heterogeneity and post-mitotic behavior of MCs [21].

In the absence of any experimental evidence regarding the origin of SCs, we attempted to understand the origin of MCC by comparing normal MCs and MCC cells based on histogenetic variations and pathological observations. Some authors suggested that MCC originates from pluripotent epidermal SCs [15, 19]. However, whether these SCs or PCs are located in the skin is still uncertain. To answer this question, we investigated the expression levels of several HFSC markers in MC and MCC cells. Based on these results, we hypothesized that both pure and combined MCC originate from HFSCs or MC PCs that reside within the HFB [27]. The presence of MCC cells within the follicular cysts suggested an association between MCC and HFs [34], which might support our hypothesis regarding the HFSC or PC origin of MCC.

We recently identified three cases of combined MCC, which led to the unexpected discovery of numerous keratin 20 (CK20)-positive MCs with normal morphology (dendritic and oval shapes) within the squamous cell carcinoma (SCC) component of combined MCC. Furthermore, one case was extremely rare due to the occurrence of an unusual combination of SCC and MCC that exhibited intra-epidermal as well as dermal neuroendocrine features. Here, we provide a detailed report of these three cases, representing the first documentation on MC hyperplasia in MCC, aside from the various benign follicular neoplasms associated with MC hyperplasia. To further uncover the pathological origin of MCC and test our hypothesis, we pathologically examined specimens of these three cases in an attempt to elucidate the proliferation potential and histogenesis of MCs and MCC cells.

Materials and methods

Clinical specimens

We performed a retrospective study by examining the histopathological and clinical data of 22 patients diagnosed with MCC, including 4 men and 18 women aged between 63 and 101 years (mean age: 83.4 years). The concurrence of MCC with an aberrant epithelial (SCC) component was considered to represent combined MCC. The retrospective protocol of this study was approved by the Saga University institutional review board. Specimens from patients with MCC were retrieved from the archives of our Division of Dermatology (Department of Internal Medicine, Faculty of Medicine, Saga University, Japan).

The tissue samples were formalin-fixed, paraffin-embedded, and stained with hematoxylin & eosin (H&E). Immunohistochemical analyses were performed by co-staining the tissue sections (thickness: 4 μm) using the following antibodies: CK20, chromogranin A (CGA), synaptophysin (SYP), or monoclonal CM2B4 antibody against Merkel cell polyomavirus (MCPyV) large T (LT) antigen using a polymer immunohistochemical detection system (Dako, Glostrup, Denmark) [1].

Morphometric analyses

To determine whether the increase in the number of MCs occurred through cell division or was generated from non-MCs, we performed double-immunofluorescence labeling to identify MCs and the active stage of the cell cycle using CK20 and Ki-67 (a proliferative marker) antibodies, respectively. We further examined the topographic distribution of CK20 and Ki-67 expression in neuroendocrine and squamous lesions in the cases of combined MCC.

The tissue sections of dermal MCC admixed with SCC were stained with antibodies against CK20 (green) and Ki-67 (red) and then counterstained with DAPI (blue) to visualize the nuclei. In the double-immunofluorescence-stained sections, MCs were identified based on a positive cytoplasmic reaction with CK20 (fluorescence isothiocyanate-channel) and evaluated only if the cell body was properly visible. During evaluation of the CK20 staining pattern, the cross-section of dendritic processes might cause misinterpretation of a dot-like pattern, which is a characteristic feature of MCC [24]. After identification, MCs were assessed for nuclear labeling using Ki-67 (Texas Red channel).

Results

Clinical manifestations in cases with combined MCC

Of the 22 specimens evaluated, we identified 10 cases of combined MCC, which were immunohistochemically MCPyV-negative, using the CM2B4 antibody. Among them, three cases exhibited a distinct increase in the number of CK20-positive cells in the co-existing SCC component, in addition to MCC cells in the combined MCC.

There were 12 cases identified as pure MCC, which did not show the characteristic feature of MC proliferation in the overlying and adjacent epidermis.

The clinical features of the three patients with combined MCC are shown in Table 1. All three patients were Japanese, female, 87–93 years old (median age: 89.6 years), and exhibited primary tumors on the face.

Histopathology and immunohistochemistry

In Case #1, the major part of the tumor exhibited epidermal replacement (complete thickness) with markedly atypical pleomorphic squamous cells (Fig. 1a–d). This corresponds with the main characteristic of SCC, which is to harbor numerous multi-nucleated cells owing to uninterrupted mitosis (Fig. 1d). In addition, the tumor was composed of intra-epidermal and dermal components, and was classified in the small blue round cell tumor (SBRCT) category by visualizing under low magnification (×40) (Fig. 1a, b).

Table 1 Basic clinicopathological characteristic of patients with combined MCC

Case #	Age	Sex	Location	MCPyV status	Aberrant component
1	93	F	Cheek	Negative	SCC
2	89	F	Temple	Negative	SCC in situ
3	87	F	Jaw	Negative	SCC

MCC Merkel cell carcinoma, *MCPyV* Merkel cell polyomavirus, *SCC* squamous cell carcinoma

The distinct proliferation of intra-epidermal and dermal cells with hyperchromatic nuclei and apoptosis were observed, which are features consistent with intra-epidermal and dermal MCCs. In addition, the proliferation features of the intra-epidermal and dermal cells revealed relatively uniform primitive SBRCT. The dermal component showed partial squamous differentiation, which was consistent with squamous differentiation of dermal MCC.

The results of histopathological and immunohistochemical analyses for this case are summarized in Table 2. Normal MCs were evaluated using normal hair follicles obtained from healthy skin specimens. After CK20 labeling but not H&E staining, distinct variations were identified in the CK20 staining patterns and cellular morphology among the dermal and intra-epidermal neuroendocrine components and among the individual cells within the SCC component.

There was variation in CK20 staining within the cells, including diffuse cytoplasmic, membranous, perinuclear, and a punctate or dot-like pattern (Fig. 1e–h).

The intra-epidermal component was positive for CK20 (lacking a characteristic perinuclear dot-like pattern), CGA, and SYP (Fig. 1f). In contrast, the dermal component was positive for CK20 (with a characteristic perinuclear dot-like pattern), CGA, and SYP (Fig. 1e). Based on these immunohistochemical characteristics, the patient was diagnosed with dermal and intra-epidermal MCC in combination with SCC.

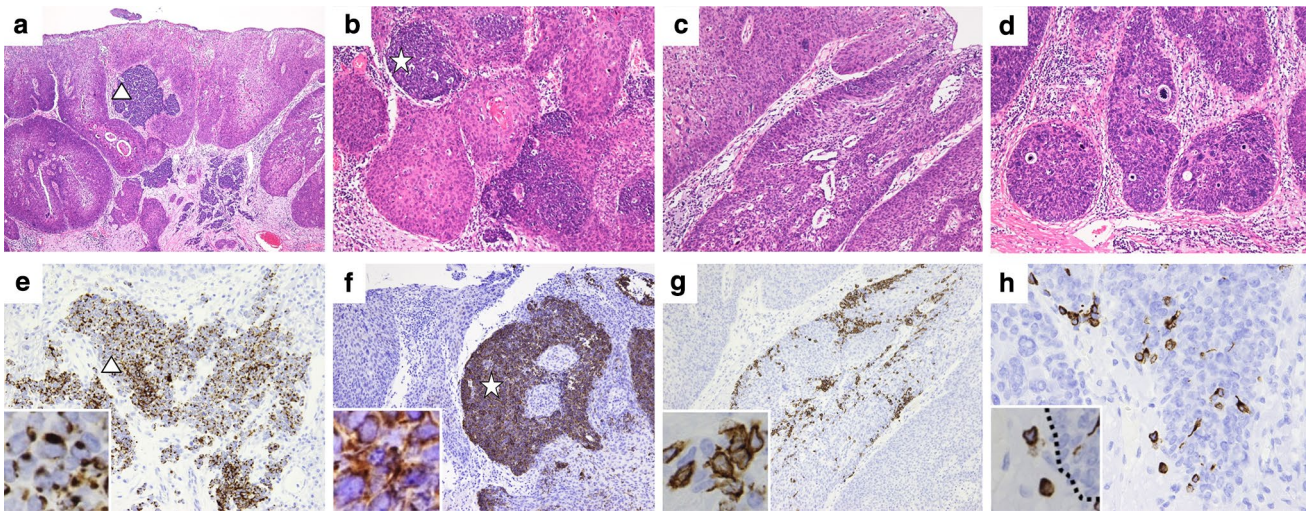

Fig. 1 Case #1: Combined MCC admixed with SCC. H&E staining (**a**–**d**) and CK20 immunostaining (**e**–**h**) images of the features displayed by the primary tumor as follows: **a** morphology of the dermal MCC (△) and SCC component; **b** morphology of the intra-epidermal MCC (☆) and SCC component; **c, d** morphology of the SCC component; **e** perinuclear dot-like pattern of CK20 immunoreactivity in most of the dermal SBRCT region (△); **f** membranous and cytoplasmic patterns of CK20 immunoreactivity in most of the intra-epidermal SBRCT region (☆); **g, h** membranous and cytoplasmic patterns of CK20 immunoreactivity in the SCC region. Characteristic perinuclear dot-like pattern of CK20 immunoreactivity is observed in (**e**) but absent in (**f**–**h**). CK20 immunoreactivity is absent in most of the SCC region, but CK20 immunoreactive cells with oval and dendritic shapes are numerous (**g**) and sparse (**h**) in the SCC region. CK20 immunoreactivity is undetectable in H&E staining (**c, d**), detectable in CK20 immunostaining, and distributed along the tumor margin (**g, h**). The inset in (**h**) displays the localization of MCs in the dermis below the basal layer (dashed line)

Table 2 Histologic and immunophenotypic variations among dermal and intraepidermal MCC, and MC hyperplasia in Case #1

	Dermal MCC	Intraepidermal MCC	MC hyperplasia	Normal MC
CK20	Dot (+)-(−)	Dot (−)	Dot (−)	Dot (−)
Chromogranin A	Positive	Positive	Positive	Positive
Synaptophysin	Positive	Positive	Positive	Positive
Histology	SBRC (+)	SBRC (+)	SBRC (−)	SBRC (−)

Dot dot-like, *SBRC* small blue round cell, *SBRC(+)* visible in HE, *SBRC(−)* invisible in HE

Interestingly, the overlying SCC component exhibited a marked increase in the number of CK20-positive cells with oval and dendritic shapes. This observation was inconspicuous during H&E staining of the histological sections, but was solely and clearly demonstrated after CK20 immunostaining (Fig. 1c, d, g, h). These CK20-positive cells exhibited cytoplasmic and membranous patterns, but not the characteristic perinuclear dot-pattern (Fig. 1g, h). In addition, CK20-positive cells revealed variable localizations as nested aggregations and solitary units within SCC (Fig. 1g, h).

Case #2 (Fig. 2a) and Case #3 (Fig. 2c) were identified as combined MCC adjacent to SCC in situ and combined MCC with overlying SCC, respectively. Immunohistochemical staining using the CK20 antibody showed cytoplasmic and membranous patterns in the CK20-positive cells within SCC in situ (Fig. 2b) and SCC (Fig. 2d). The CK20-positive cells lacked the perinuclear dot-like patterns, exhibited oval

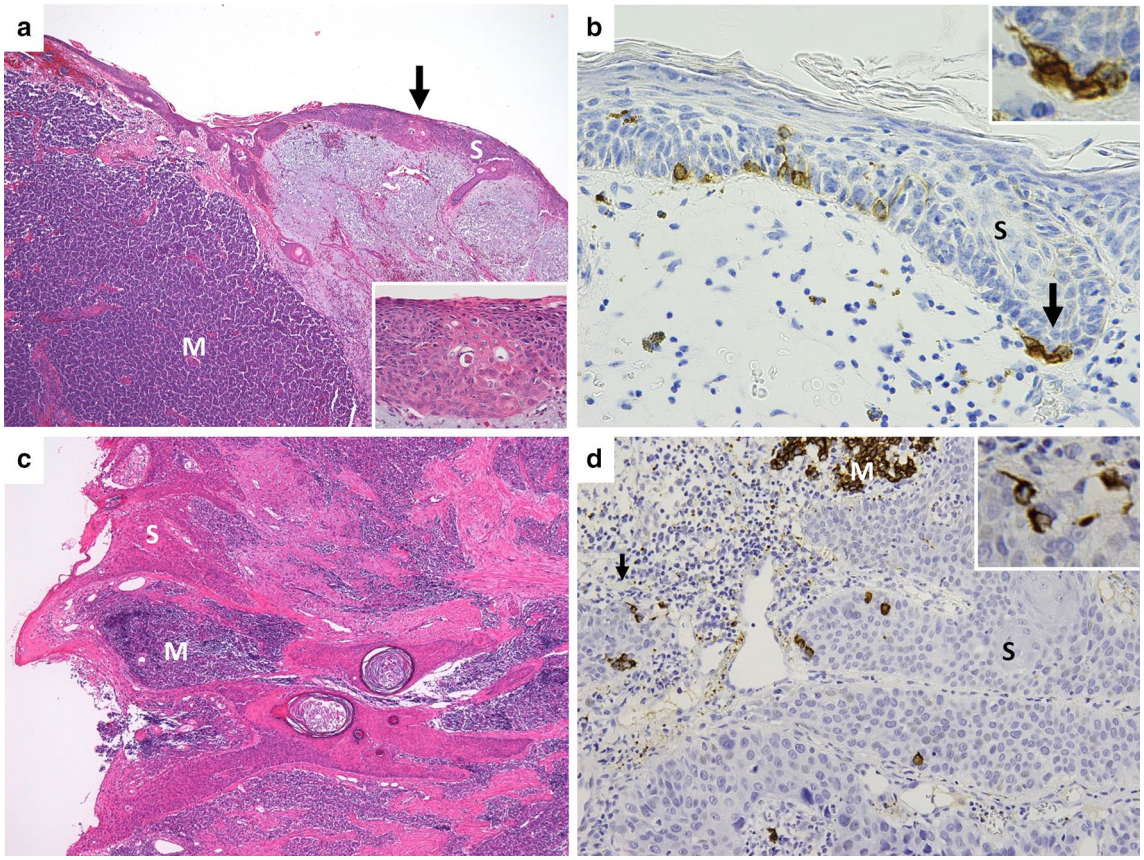

Fig. 2 H&E staining shows the histological features of combined MCC adjacent to SCC *in situ* (Case #2) and overlying SCC (Case #3). The inset in **a** shows the high-magnification image of the area pointed by the arrow-head (×200). Atypical squamous cells proliferation is seen throughout the thickness of the epidermis. Immunohistochemical staining using the CK20 antibody shows cytoplasmic and membranous patterns in CK20-positive cells within SCC in situ (Case #2; **b**) and SCC (Case #3; **d**). The inset in **b, d** shows the high-magnification image of the area pointed by the arrow (×400). CK20-positive cells lack perinuclear dot-like patterns, exhibit oval and dendritic shapes similar to normal MCs, and are localized in the basal layer

and dendritic shapes similar to normal MCs, and were localized in the basal layer.

Normal MCs were predominantly found in the basal layer of the epidermis and occasionally in the skin surrounding the hair follicles (HFs). The CK20-positive cells with round and dendritic shapes were morphologically indistinguishable compared to normal MCs at the single-cell level.

Comprehensively considering the above findings, we interpreted the proliferation of CK20-positive cells to be MC hyperplasia and not MCC.

Double-immunofluorescence labeling using Ki-67 and CK20 antibodies

Double-immunofluorescence staining with antibodies against CK20 (green) and Ki-67 (red) demonstrated a considerable degree of co-expression (Fig. 3). In addition, there was substantial variation in the incidence frequency of CK20 and Ki-67 co-expression between neuroendocrine and SCC components.

Ki-67/CK20 double-positive MCs (DPMCs) were detected and represented approximately 90% of the cells in the dermal neuroendocrine component (Fig. 3c). However, in the SCC component, DPMCs accounted for a relatively high frequency (50%) to only a minority (15%) of the cells (Fig. 3f, i).

Considering that CK20-positive cells were undetectable using H&E staining and were solely detectable using CK20 immunostaining, the Ki-67/CK20 DPMCs revealed the proliferation ability of MCs, but not MCC cells, in cases of combined MCC.

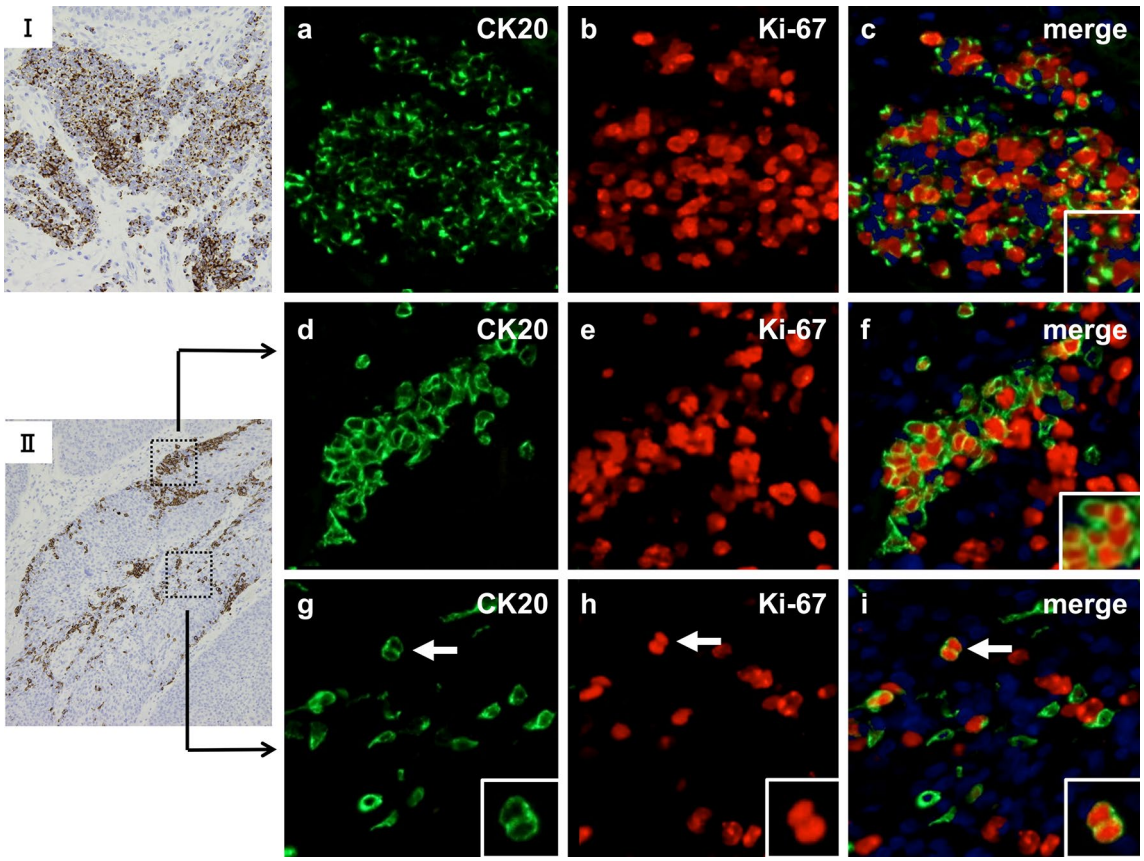

Fig. 3 CK20 immunostaining to identify the lineage of proliferating cells in combined MCC composed of MCC (I) and SCC (II). Images of double-immunofluorescence staining using antibodies against CK20 and Ki-67 to display the proliferation ability of CK20-immunoreactive cells in combined MCC. Staining of CK20 (green) alone (**a, d** and **g**), Ki-67 (red) alone (**b, e**, and **h**), and CK20 and Ki-67 in combination (merge) (**c, f**, and **i**). **a, c** accumulation of perinuclear dot-like patterns in CK20-positive cells at the dermal MCC; **d, f** accumulation of cytoplasmic and membranous patterns in CK20-positive cells at the SCC; **g, i** sparse distribution of cytoplasmic and membranous patterns in CK20-positive cells at the SCC. Double-positive cells represent considerable variation in the incidence frequency of CK20 and Ki-67 co-expression (**c, f, i**). The CK20- and Ki-67-positive replicating MC is indicated by arrows in (**g–i**). Insets represent the high-magnification images of the respective main images

Discussion

In the current study, we visualized proliferating MCs by performing double-immunofluorescence staining using mouse monoclonal antibodies against CK20 and Ki-67 in MCC combined with SCC. Besides the detailed histopathological features of the extremely unique case of coexisting intra epidermal and dermal MCC with SCC, the main novelty of these findings is the first demonstration, to the best of our knowledge, of the proliferating potential of MCs during specific conditions and its implications in the histogenesis of MCC.

In the present study, immunohistochemical analysis of CK20 expression in Case #1 demonstrated a characteristic dot-like pattern of the dermal MCC, whereas the intra-epidermal MCC lacked a perinuclear dot-like pattern, and instead exhibited cytoplasmic and/or membranous patterns, hence suggesting that dermal and intra-epidermal MCCs originate through distinct tumorigenesis mechanisms.

The intra-epidermal proliferation of MCs might lead to a diagnostic quandary. However, the intra-epidermal proliferation of MCs without the dermal component was interpreted as either a hyperplastic or a neoplastic process, namely, MCC in situ.

In the present study, MCs presented as clusters and solitary units in the SCC component and did not display the morphology of SBRCT in the H&E-stained sections. However, the cells exhibited round and dendritic shapes with long dendritic processes, and the absence of mitosis, apoptosis, and the characteristic perinuclear dot-like pattern of CK20 staining. These results indicated a hyperplastic process of morphologically normal MCs, but not a neoplastic process. However, a limitation of our study was that we were unable to distinguish between normal MCs and neoplastic MCCs since both populations expressed CK20.

Although the frequency of incidence was low, CK20-positive MCs in the SCC component were also positive for Ki-67. This result may be interpreted to indicate that the hyperplasia of MCs was caused by replication of CK20-positive differentiated MCs, MCPCs, or dedifferentiated MCs that reenter the cell cycle and replicate. In addition, our findings indicate that MCs should not be considered as pure post-mitotic cells since they could potentially proliferate during specific conditions, such as in diseased or wounded skin, or at sites adjacent to various skin tumors, including MCC.

In case #1, proliferating MCs within the SCC component were negative-stained with p53 (data not shown). In studies regarding their cell-cycle properties, this finding may suggest the possibility that abnormalities in p53 pathway confer normal MCs a growth advantage. However, since it is highly speculative, further studies are recommended to clarify the significance of p53 in the biology of MCs.

In conclusion, based on the present histogenetic variations and pathological observations of three cases of combined MCC, we postulate that MCs and MCC cells might originate from normal and transformed MCPCs that reside in the HFB. The topographical symbiosis of MCC and MC hyperplasia may implicate a common PC. The intimate coexistence of two types of malignant cells, namely dermal and intra-epidermal MCC cells, and morphologically normal proliferating MCs, in the same lesion, supports the concept that MCs and MCC cells are derived from MCPCs, which exhibit the potential to differentiate (dual-directional) into both neuroendocrine or epithelial lineages. Therefore, we hypothesize that MCs and MCC cells are derived from normal and transformed MCPCs, respectively.

Acknowledgements This study was supported by JSPS (the Japan Society for the Promotion of Science) KAKENHI [Grant number 16K10164].

Conflict of interest The authors declare that they have no conflict of interest.

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