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Plasticity of neuropeptidergic neoplasm cells in the primary and metastatic Merkel cell carcinoma

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Abstract: Merkel cell carcinoma (MCC) is a rare and highly aggressive cutaneous carcinoma with characteristics of neuroendocrine tumor. We performed immunohistochemical analysis to demonstrate the presence of various neuropeptides within cells of MCC resected from a 75-year old woman. The cells of primary tumor of cheek were compared with the cells of regional right submandibular metastatic tumor which was found eight months later. A double-staining IHC for the pan-neuronal marker, PGP 9.5, and selected neuropeptides in the tissue material obtained from both locations was performed. Single multipolar cells in the main mass of primary tumor stained positively for PGP 9.5 and such neuropeptides as GAL, VIP, PACAP, NPY and CGRP. Moreover, we demonstrated for the first time the presence of neuropeptides in metastatic MCC cells. In the metastatic tumor, cells showing the co-localization of PGP-9.5 and neuropeptides were more numerous, mostly of oval shape, and significantly smaller than in the primary tumor. Thus, the progression of MCC may be associated with the acquisition by its cells of new morphological and biological features. *(Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 2, 168–173)*

Key words: merkel cell carcinoma, metastasis, GAL, VIP, PACAP, NPY, CGRP, PGP-9.5

Introduction

Merkel cell carcinoma (MCC) is a rare and highly aggressive cutaneous tumor. Its true origin has not been proved definitively. The presence of electron-dense neurosecretory granules in the tumor cells led to its classification among the neuroendocrine carcinomas. As Merkel cells, which belong to the amine precursor uptake and decarboxylation system (APUD), are the only cutaneous cells that possess such granules, it is believed that MCC is derived from these cells [1]. The numerous secretory vacuoles of Merkel cells contain many biologically active substances such as vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gen-related peptide (CGRP), galanin (GAL) and neuropeptide Y (NPY) [2–6]. Moreover, Merkel cells stain positively for the nervous and neuroendocrine cell markers such as protein gene-product 9.5 (PGP 9.5) and chromogranin A [7, 8]. Interestingly, in primary MCC the presence of some neuropeptides was demonstrated only in a part of investigated tumors [9–10]. Since the presence of neuropeptides in the metachronous metastatic MCC tumor as well as the

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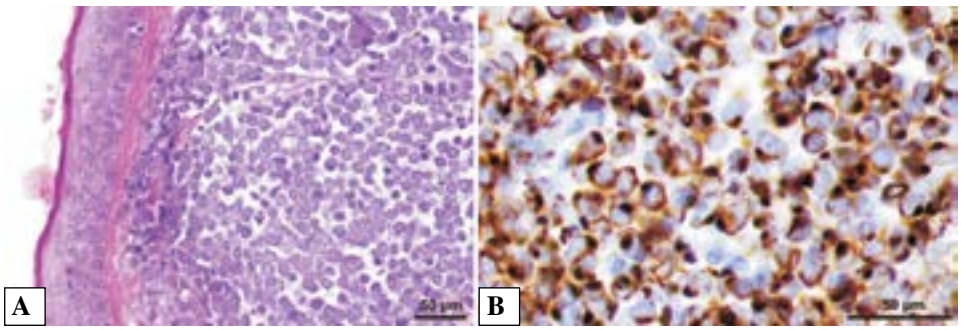

Figure 1A. Section through the MCC tumor of the lower eyelid stained with H&E. Neoplasm was composed of a mass of small and oval cells; epidermal coating of the tumor is also visible. × 200; **B.** Section stained with primary antibody against CK 20 (MCC marker) demonstrated the presence of this cytokeratine type in the neoplasm; characteristic reaction is present in the cytoplasm of cancer cells. × 400

comparison to the primary MCC tumor has not yet been investigated, the aim of this study was to perform such immunohistochemical analyses in a single case of MCC.

Material and methods

MCC tissue was obtained from a 75-year-old female who had the skin tumor of the upper part of right cheek/lower eyelid. Detailed clinical examinations revealed lack of any others diseases. The tumor was removed within the margin of macroscopically healthy tissues, and the loss of skin was reconstructed with a rotation flap. Macroscopically, it was a pale-cream colored tumor with the dimensions of 1.6×1.6×2 cm, covered with epidermis. It was diagnosed by pathologist on the basis of the examination in hematoxylin-eosin (H&E) stained slides (Figure 1A) as a small cell type of MCC (small cell type of MCC), with immunohistochemical characteristics such as the presence of cytokeratine 20 (CK20)/+/ (Figure 1B), and CD56/+/, chromogranin /+/, synaptophysin/+/ (not shown). The rapid recurrence within the postoperative scar, after 6 weeks from the first operation was observed. The recurrence was excised and the extensive loss of the skin was supplemented with plastics by free flap of the skin.

In the course of further observation, local recurrence was not observed within this region, however, after 8 months the metastatic tumour grew in the right submandibular area. For this reason, the patient underwent surgery and the tumor was removed. The histopathological assessment confirmed the metastasis of MCC with the same histological and immunohistochemical characteristics as the primary tumor.

Immunofluorescence analysis. To assess the presence of neuropeptides, after histopathological assessment of the primary and metastatic, serial paraffin sections were obtained and double-stained by immunofluorescent technique to check for the presence of PGP 9.5, and several neuropeptides (VIP, PACAP, NPY, CGRP, GAL). For this purpose following primary antibodies were used: mouse anti-PGP–9.5, dilution 1:2000 (AbD Serotec, Kidlington, UK); rabbit anti-galanin, dilution 1:2000; rabbit anti -PACAP, dilution 1:10000 (Peninsula Laborat.Inc., San Carlos, CA, USA); rabbit anti-CGRP, dilution 1:8000 (SigmaAldrich, St. Louis, MO, USA); rabbit anti-NPY dilution 1:5000 (Biomol, Farmingdale, NY, USA); rabbit anti-VIP, dilution 1:5000 (Abcam, Cambridge, UK). The secondary antibodies used were goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555, both in dilution 1:500 (Invitrogen, Eugene, OR, USA). Control slides were processed without the use of primary antibodies. The immunofluorescent reactions were evaluated in sections with the use of a confocal microscope (Zeiss LSM 710, Carl Zeiss MicroImaging, Wetzlar, Germany). Moreover, cell size was measured with the use of Zen 2011 program (Carl Zeiss MicroImaging, Wetzlar, Germany).

Results

The microscopic observations of primary tumor sections showed the presence of single cells stained towards PGP-9.5 (Figure 2–5A) and the examined neuropeptides PACAP (Figure 2B), CGRP (Figure 3B), GAL (Figure 4B), VIP (Figure 5B), and NPY (not shown). These cells were scattered throughout the tumor tissue and due to their size and clearly visible protrusions, they were easily recognizable in the main tumor mass which consisted of smaller, oval and immunofluorescence-negative cells. The neuropeptide-immunoreactive cells exhibited two or three processes widely branching from the cell body, suggestive of the multipolar cell shape. Within the cytoplasm of these cells granules containing neuropeptides were visible. The average diameter of the primary tumor cells was 20.5 ± 2.7 μm (mean SD, range 14–26 μm).

In the metastatic tumor, cells showing the co-localization of PGP-9.5 (Figure 2–5D), PACAP (Figure 2E),

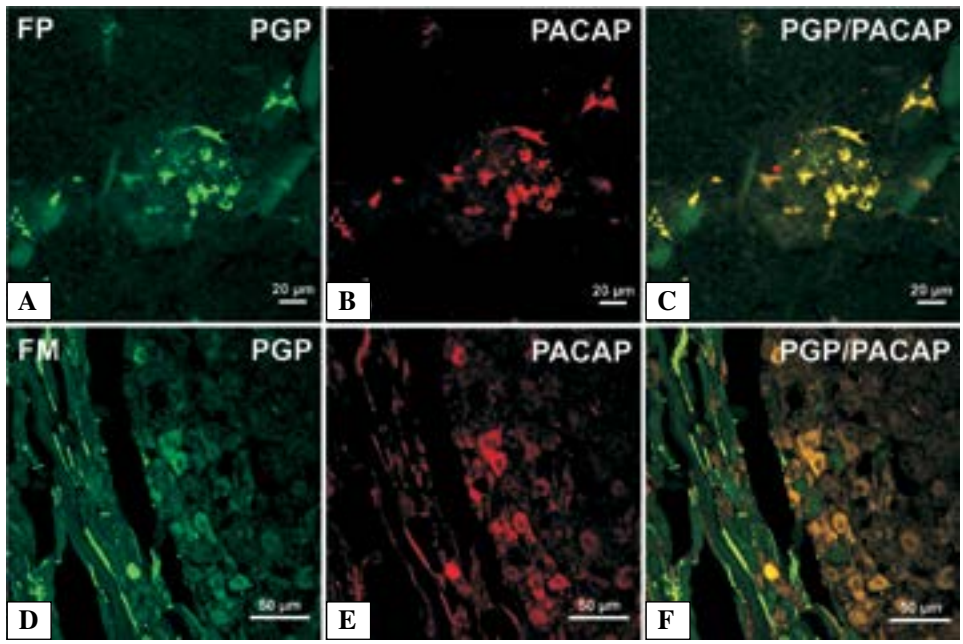

Figure 2. Sections of primary (FP, **2A–C**) and metastatic (FM, **2D–F**) MCC stained against PGP-9.5 (**2A. D**) and PACAP (**2B, E**) were examined by confocal laser scanning microscope (CLSM). Superimposition (**2C, F**) revealed double-labeled yellow/orange cancer cells. Multipolar cancer cells in primary tumor show cytoplasmic granules containing PACAP, however, in metastatic tumor many oval neuropeptide-containing cells predominate. Bars: **2A–C**, 20 μm; **2D–F**, 50 μm

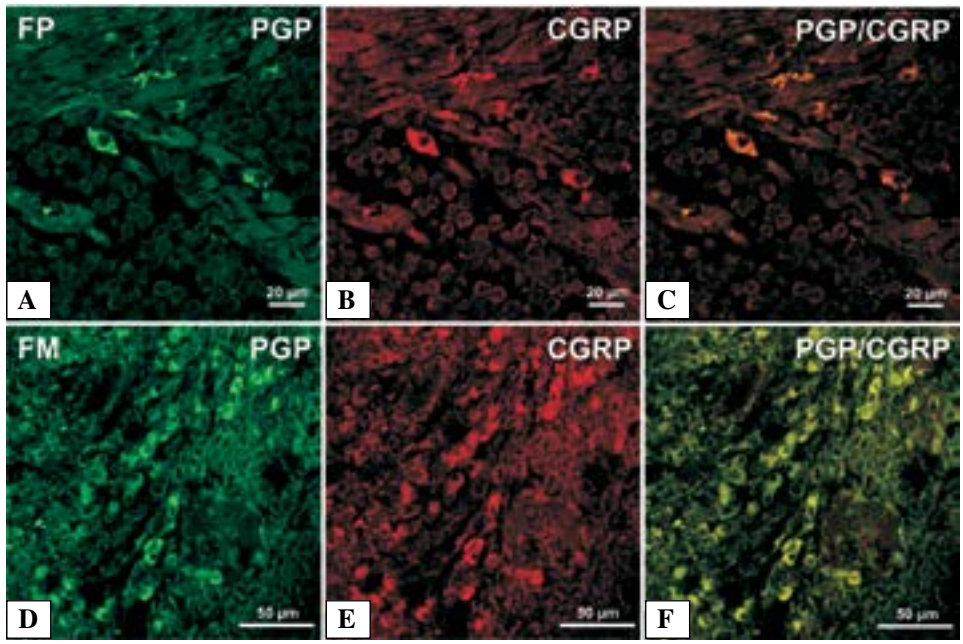

Figure 3. Sections of primary (FP, **3A–C**) and metastatic (FM, **3D–F**) MCC stained against PGP-9.5 (**3A, D**), CGRP (**3B, E**) and double-labeled (**3C, F**). Difference in number, size and shape of metastatic cancer cells in comparison to cells of primary tumor is evident. Bars: **3A–C**, 20 μm; **3D–F**, 50 μm

CGRP (Figure 3E), GAL (Figure 4E), VIP (Figure 5E) and NPY (not shown) were observed. Immunoreactive cells were more numerous than those in the primary tumor, located closer to each other and grouped in clusters. Most of the cells were oval; however, slightly elongated cells were also visible. The cells which showed neuropeptide immunoreactivity were smaller than multipolar immunopositive cells found in the primary tumor. An average, diameter of these cells was 13.18 ± 1.9 μm (range 10–19 μm).

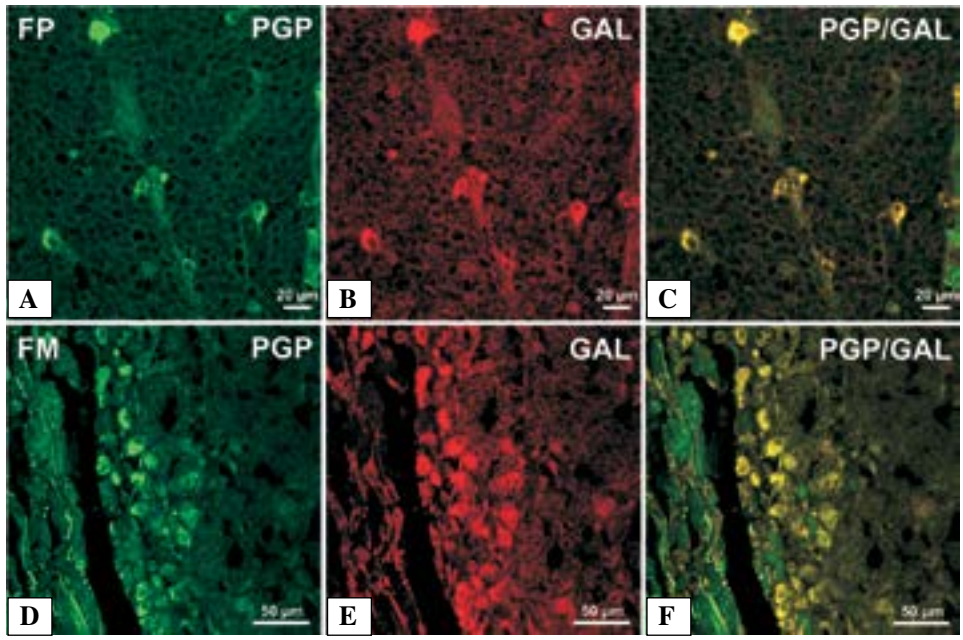

Figure 4. Sections of primary (FP, **4A–C**) and metastatic (FM, **4D–F**) MCC stained against PGP-9.5 (**4A, D**), GAL (**4B, E**), and double-labeled (**4C, F**). In primary tumor few cancer cells express GAL, but in the metastatic tumor GAL (+) neoplastic cells are considerably more numerous and located close to each other. Bars: **4A–C**, 20 μm; **4D–F**, 50 μm

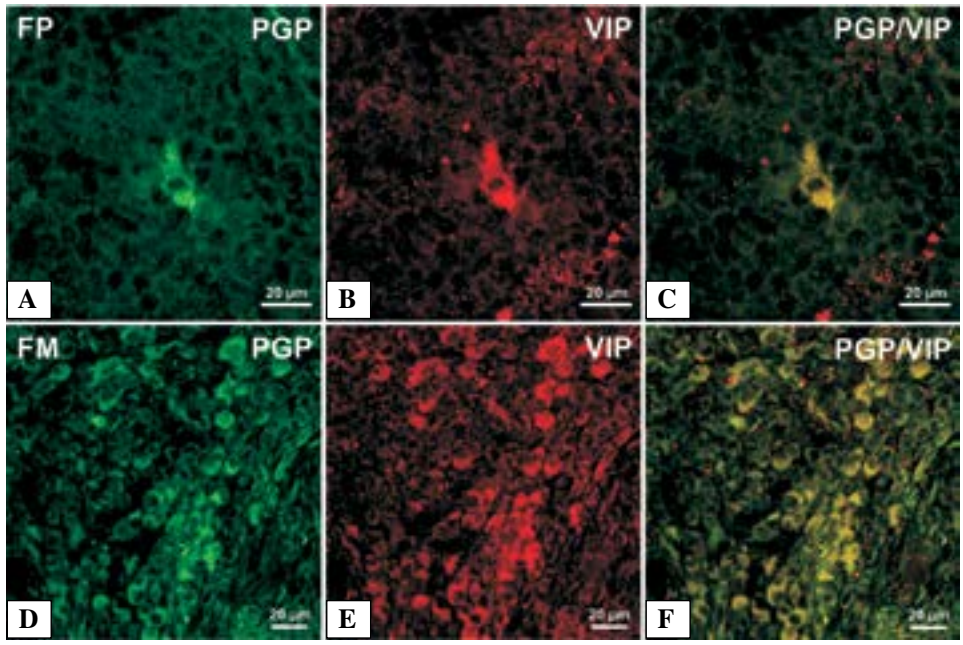

Figure 5. Sections of primary (FP, **5A–C**) and metastatic (FM, **5D–F**) MCC stained against PGP-9.5 (**5A, D**) and VIP (**5B, E**) and double-labeled (**5C, F**). In primary tumor only one cancer cell is visible within tumor mass, whereas in metastatic tumor cancer cells are more numerous, smaller and of oval shape. Bars: **5A–C**, 20 μm; **5D–F** 50 μm

Discussion

The origin and etiology of MCC are still controversial. There are differences and similarities between Merkel cells and the cells of MCC. Although Merkel cells are abundant in areas of skin involved in touch perception (*e.g.* finger tips), this location is uncommon for MCC. Moreover, the cells of MCC do not express the opioid peptides identified in normal Merkel cells [11]. On the other hand, ultrastructural studies revealed the presence of dense-core neuroendocrine granules in the cytoplasm of tumor

cells similar to those observed in Merkel cells, thus suggesting association between the neoplasm and the normal neuroendocrine cell population of the skin Merkel cells [12]. An immunohistochemical investigation performed by Fantini and Johansson [6] revealed expression of CGRP, VIP, peptide histidine isoleucine amide, NPY, neurokinin A, GAL, SP, and somatostatin (SOM), in human cutaneous Merkel cells. These immunoreactivities were very variable in terms of the number of positively stained cells and intensity of staining, indicating that a complex and heterogeneous immunophenotype can be expressed within this population of cells. Halata et al. [2] distinguished two groups of Merkel cells and suggested that Merkel cells with associated nerve terminals function as mechanoreceptor cells, while cells without contact to nerve terminals belong to the diffuse neuroendocrine system (DNES), do not function as mechanoreceptors, and are the origin of MCC. Results of previous investigations of the presence of neuropeptides in MCC cells are unequivocal. Some MCCs were reported to show positive reactions for VIP, SP, and SOM [13], however, Furuno et al. [14] did not observe neuropeptides in the MCC of the eyelid. Our findings revealed the presence of single cells stained towards GAL, VIP, PACAP, NPY and CGRP in primary tumor and significantly more numerous immunopositive cells in the metastatic tumor tissue. These differences may be caused in part by the fact that the distribution of neuropeptides varies depending on the body region. High levels of immunoreactivity of SP, neurokinin A and CGRP in Merkel cells are found in areas with the greatest tactile sensation, intermediate levels in neck and face, whereas the lowest immunoreactivity was observed in Merkel cells in groins, arms, and thighs [15]. To our knowledge, there are as yet no reports analyzing the location of neuropeptides in metastatic MCC and comparison of their presence in neoplastic cells of the primary and metastatic tumors of the same patient. The differences in the morphology of immunoreactive cells observed by us in the primary and metastatic tumors may be related to the histological types of MCC. So far several histologic types are recognized in MCC based on the size of the cells (small, intermediate, large) or on the tumor's architecture (solid, diffuse, or trabecular type) [16]. A large-cell type often has a trabecular pattern and a high density of granules at the ultrastructural level, while the ultrastructure of a small-cell type reveals paucity of granules and resemblance to skin metastases of small-cell lung carcinoma [17]. In contrast to a study by Sandel et al. [18], Skelton et al. [19] observed a correlation between small cell type predominance and poor prognosis. The small cell growth pattern is a characteristic feature of a one subtype of MCC invasion. The small cell morphology was found to be associated with high biological malignancy, fast growth, as well as early formation of metastases [20].

Neuropeptides can function as autocrine growth factors in cancer cells. Previous studies confirmed presence of neuropeptides in different types of cancers, *e.g.* PACAP in prostate and breast cancer or VIP and NPY in prostate and urinary bladder cancer [21–23]. Further investigations established the presence of neuropeptide receptors, *e.g.* VPAC1, VPAC2 and PACAP receptors, respectively, in prostate, breast and lung cancer tissues [24–28]. The occurrence of neuropeptides and their receptors within tumor may strongly suggest an autocrine pattern of signaling resulting in a faster tumor growth. In the small cell lung cancer (SCLC), stimulation of VPAC1 and PAC1 receptors increased the levels of intracellular cAMP, as well as activated tyrosine kinase cellular pathways [29]. It was also shown that VIP caused the release of BB (bombesin)-like peptides from SCLC cells, which in an autocrine manner, *via* BB2 bombesin receptors and mitogen activated protein kinase (MAPK) pathway increased expression of cell growth factors [30]. Moreover, an oncogenic impact of GAL receptor via elevation of intracellular ionized calcium concentration was also found in SCLC [31]. Furthermore, it was shown that CGRP increased the invasive potential of prostate cancer cells partially by the enhancement of cell motility [32]. The previously described presence of neuropeptides and their receptors within Merkel cells of a normal epidermis may suggest a possible autocrine function of the neuropeptides in Merkel cells [5].

Microscopic observations carried out within this study for the first time showed the presence of GAL, VIP, PACAP, NPY and CGRP within cells of not only primary but also metastatic MCC. We also observed neuropeptidergic plasticity of the neoplastic cells comparing these two MCC locations. The increase in the number of phenotypically different neuropeptide-containing cells in metastatic MCC suggests that the neuropeptides may accelerate tumor's growth and its invasiveness.

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