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Neuroendocrine Differentiation in Primary Merkel Cell Carcinoma – Possible Prognostic Significance

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Abstract. *Background: The aim of this study was to examine the level of neuroendocrine differentiation to determine its association with clinicopathological parameters. Patients and Methods: Twenty-five primary MCC samples were evaluated for neuroendocrine differentiation profiles by immunohistochemistry using antibodies to chromogranin-A, microtubule associated protein-2 and synaptophysin. The data were compared with clinical parameters to find out whether their expression correlates with prognosis. Results: In general, MCC shows a high degree of neuroendocrine differentiation. A higher expression of chromogranin-A and synaptophysin associated with benign behaviour. Chromogranin-A appeared to be the most important one in predicting the course of disease. Conclusion: Low levels of neuroendocrine differentiation in MCC associates with poor prognosis. Chromogranin-A could be used to identify patients who might benefit from oncological treatments.*

Merkel cell carcinoma (MCC), also referred to as primary neuroendocrine carcinoma, arises from neuroendocrine Merkel cells in the dermis with features of epithelial differentiation. It belongs to a group of highly malignant tumours known as small blue cell tumours, which share a common histologic characteristic with haematoxylin-eosin staining (1).

MCC is a rare and potentially aggressive tumour that has a poorer prognosis than other epidermal skin tumours. MCC's biological behaviour includes rapid progression of the primary tumour, with common local recurrences as well as early and frequent metastasis to local lymph nodes and even systemic metastasis (2-4). MCC arises mainly on sun-exposed areas of the skin, specially in the head and neck of people over 60 years of age (5). Of the prognostic factors, large tumour size, male gender and older age have been found to have a negative impact on overall survival (6, 7).

MCC, as well as normal Merkel cells, express antigens to cytokeratin-20 (CK-20) and epithelial membrane antigen (8-10). CK-20 it is not expressed in neuroendocrine carcinomas of other sites, such as small cell lung carcinoma (11). Therefore, CK-20 immunostaining is widely accepted for the diagnosis of MCC (12). Thyroid transcription factor-1 (TTF-1) immunostaining is used for differentiation between MCC and small cell lung carcinoma metastasis (13).

Neuroendocrine differentiation in MCC has been described previously in several reports; MCC stains positively for neuron-specific enolase, a general marker of neuroendocrine tumours (14). CD56, or neural cell adhesion molecule, has recently been demonstrated to be a neuroendocrine marker of the pulmonary neuroendocrine cell system as well as MCC (15). Also, expression of neurofilaments are common (16, 17). Mount and Taatjes have previously demonstrated cytoplasmic synaptophysin (SYP) and chromogranin A (Cr-A) positivity over dense-core granules to exemplify the neuroendocrine differentiation in MCC (18).

Cr-A is a widely distributed marker for endocrine tumours. It was first isolated from chromaffin cells of the adrenal medulla (19). MCC shows focal, limited positive immunoreaction for Cr-A (20). SYP a transmembrane channel protein of small presynaptic vesicles, is expressed in neuroendocrine and neural cells and diffusely in the neuroendocrine system (21). MCC shows consistently positive immunoreaction for SYP (22). Quite recently, a new marker for neuroendocrine differentiation has been introduced for neuroendocrine carcinomas and, thus, also for MCC; microtubule-associated protein-2 (MAP-2) is part of a group of polypeptides that builds up the microtubular cytoskeletal structure of the central and peripheral nervous

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Key Words: Primary neuroendocrine carcinoma, Merkel cell carcinoma, neuroendocrine differentiation, prognosis, chromogranin-A.

Table I. *Patient and disease descriptions of the 25 MCC patients.*

N	25
Age (years)	76.7 range 59-100
Gender (M/F)	10/15
Tumour size (cm)	2.5 range 0.8-6.5
Location	
head and neck	11
trunk	3
extremities	11
Local recurrence	8
Metastasis	11

system. Immunoreactivity of MAP-2 has been demonstrated in most neuroendocrine and neuroectodermal-related neoplasms such as small cell carcinoma, large cell neuroendocrine carcinoma, carcinoid tumour of the lung, Merkel cell carcinoma of the skin, medulloblastoma, neurocytoma of the central nervous system, extrapulmonary small cell carcinoma and carcinoid tumour, and malignant melanomas (23). Particular MAPs have been identified in specific cell types (24). MAP-2 is a very sensitive and specific marker for neuroendocrine differentiation (23, 25). In MCC, MAP-2 is expressed in a focal to diffuse pattern (26).

The aims of this study were to study the expression level of three common neuroendocrine differentiation markers, Cr-A, MAP-2 and SYP, by immunohistochemistry, to establish potential clinicopathological correlations of neuroendocrine markers expression in primary MCC and, further, to determine the prognostic significance of different patterns of neuroendocrine markers expression.

Patients and Methods

Patients. The study comprised 25 patients treated for MCC between 1987 and 2003 at the Department of Plastic Surgery, Helsinki University Central Hospital and at Vaasa Central Hospital, Finland. One primary tumour sample was collected from each of the patients. In haematoxylin-eosin (HE) staining, typical histology was small blue cells with sparse cytoplasm. Nuclei were medium-sized. Mitoses were seen abundantly and the tumour expanded frequently to subcutaneous tissue. The diagnoses were confirmed with immunohistochemical analysis using CK-20 and TTF-1 antibodies, the latter being negative in all samples. Tumour size (the greatest surface dimension) was measured from HE- stained slides and documented as <2 cm or ≥ 2 cm, this cut-off point being chosen based on our previous study results (6). None of the patients received chemo- or radiation therapy preoperatively. Clinical outcome was recorded from the hospital records. Table I presents an overview of the patient descriptions.

For the immunohistochemical analysis for MAP-2 and SYP we were able to get 23 samples and for Cr-A 24 samples. Immunohistochemical analyses were performed and the results were statistically compared with tumour characteristics and clinical outcome.

Methods. Five-micrometer sections were cut from the paraffin blocks and mounted on charged slides. The sections then were deparaffinised, rehydrated and treated with 1% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. The sections then were rinsed and incubated with primary antibodies against MAP-2 (Sigma, St.Louis, MO, USA), Cr-A (Sigma) and SYP (SYN Sigma). The immunostaining was performed on an automated immunostainer with appropriate positive and negative controls.

One section of each tumour was analysed, and the staining pattern was recorded. For quantification of immunostaining, we used the method described earlier by Huuhtanen and co-workers (27). This was done by choosing a tumour area with the highest density of positive nuclear staining. An ocular grid of 100 (10 x 10) squares was used at 10x40 magnification to calculate the percentage of positively-stained nuclei. Five representative high-power fields were chosen, and the distribution of immunoreactivity was analysed by quantifying the percentage of positive cells, which was expressed as the labelling index (LI). The results were scored by two researches (TB and VK). The intensity of the immunoreaction was recorded as negative (-), low (+), moderate (++) or strong (+++). A sample of normal pancreatic tissue with Langerhans islands served as a positive control to minimize interassay variation. Negative controls consisted of incubations with the omission of primary antibodies.

Statistical analysis. Statistical analysis was done with NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA) software. The correlatios between the quantitative expression of Cr-A, MAP-2 and SYP and tumour size, tumour location, local recurrence and metastatic dissemination were calculated by the Chi-square test. The level of significance was chosen as $p<0.05$, unless otherwise noted. The local ethics committee approved the study protocol.

Results

Positive staining for Cr-A was seen in all 24 samples, positive staining for MAP-2 in 22 of the 23 samples and positive staining for SYP in 22 of the 23 samples. The two completely negative stainings, one for MAP-2 and one for SYP, were in tumours which expanded to metastatic dissemination during the course of disease. The staining intensities and mean labelling indices (LI) of 25 primary MCC samples are presented in Table II. The staining patterns were focal, except in two samples: one in MAP-2 and one in SYP, where staining positivity was seen covering the whole sample. Both these patients expanded to metastasis. The patient who had both MAP-2 and SYP positivity in the whole sample had simultaneously low Cr-A positivity. She presented with systemic dissemination at presentation.

Table II *Immunohistochemical expression of chromogranin-A (Cr-A), microtubule-associated protein-2 (MAP-2) and synaptophysin (SYP) in 25 primary MCC samples.*

Sample number	Cr-A Intensity	LI	MAP-2 Intensity	LI	SYP Intensity	LI
1	++	20	+++	30	+++	90
2	+++	80	+	10	++	90
3	++	30	+	20	++	80
4	+++	80	++	80	+++	90
5	++	50	+++	50	++	80
6	+++	80	+++	90	+	80
7	+	5	+++	90	++	90
8	+++	80	NP	NP	NP	NP
9	+++	90	++	80	+++	90
10*	+	10	-	0	+	90
11*	++	10	+++	90	+	70
12*	+	5	+	5	-	0
13	++	80	+	5	+	80
14	++	80	+	5	+	80
15*	+++	80	NP	NP	NP	NP
16	++	80	+++	80	+++	90
17	NP	NP	++	80	++	80
18*	+++	30	+++	100	+	10
19*	+++	50	+++	80	+++	80
20*	+	20	+++	100	+++	100
21*	++	50	+	5	++	80
22*	++	80	+++	60	++	80
23*	+	10	++	50	+	90
24*	+++	80	+++	50	+++	80
25	++	5	+++	80	++	80

Intensity: negative (-), low (+), moderate (++) or strong (+++). NP not performed. * Patient expanded to metastasis. LI, labelling index i.e. percentage of positive cells compared to the whole tumour's cellularity.

In general, Cr-A expression varied between 5 – 80% (mean LI 48.9%), MAP-2 expression between 5 – 100% (mean LI 53.9%) and SYP expression 10 – 100% (mean LI 77.8%). The intensities in Cr-A expression were low in 10 (41.6%), moderate in 9 (37.5%) and strong in 5 (20.8%). MAP-2 intensities were negative in 1 (4.3%), low in 6 (26%), moderate in 4 (17.4%) and strong in 12 (52.2%). The intensities for SYP were negative in 1 (4.3%), low in 6 (26%), moderate in 8 (34.7%) and strong in 8 (34.7%), Figure 1. presents examples of the immunohistochemical staining. Table III. illustrates the LIs and staining intensities samples expanding to metastases and according to the tumour size.

Statistical analysis revealed that the intensity of SYP immunohistochemical expression correlated with head and neck location of the primary tumour, $p=0.017$. Tumours with low intensity Cr-A expression metastasised often, but the difference was statistically insignificant ($p=0.08$). The intensity of Cr-A staining correlated with tumour invasion to the underlying subcutaneous tissue, in a way with tumours with lower staining intensities seemed to invade to the subcutaneous tissue more often, ($p<0.09$). In addition, lower staining intensities in Cr-A associated with tumour recurrence ($p=0.07$), but then again, this was statistically non-significant.

Discussion

The degree of neuroendocrine differentiation in 25 primary MCC samples was examined. Neuroendocrine differentiation was studied by using three markers, namely chromogranin A (Cr-A), synaptophysin (SYP) and microtubule-associated protein-2 (MAP-2). Positive staining for Cr-A was apparent in all 24 samples (100%), whereas MAP-2 and SYP expression was evident in 22 (95%) of the 23 samples. Earlier, Haneke and co-workers had demonstrated similar results in Cr-A immunohistochemical analysis; 80% of their MCC samples showed a positive reaction for Cr-A (20). Liu and co-workers confirmed 100% positivity in their MCC samples in MAP-2 immunohistochemical analysis (26). In general, the expression of the antibodies tested in this study varied between 0 – 100%, the mean LI being high; Cr-A 49%, MAP-2 54% and SYP 78%. Staining intensities were

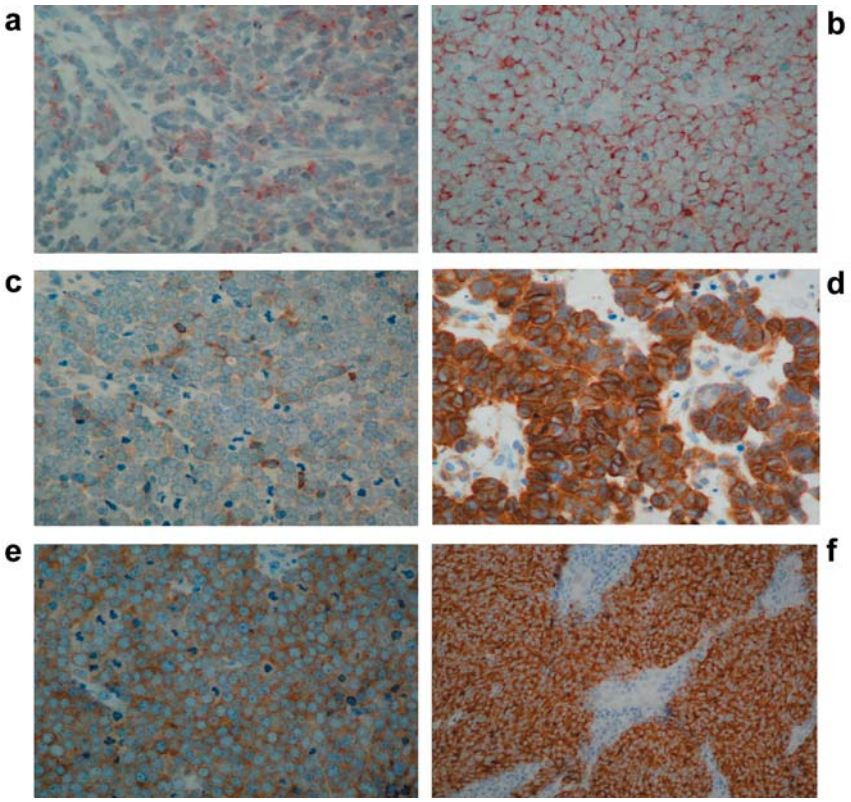

Figure 1. *Immunohistochemical staining of primary MCC for neuroendocrine differentiation. A. Low intensity (+) Cr-A staining, LI 20%, original magnification 200x. B. Strong (+++) intensity Cr-A staining, LI 80% original magnification 200x. C. Low (+) intensity MAP-2 staining, LI 10%, original magnification 200x. D. Strong (+++) intensity MAP-2 staining, LI 90%, original magnification 400x. E. Moderate (++) intensity SYP staining, LI 80%, original magnification 200x. F. Strong (+++) intensity SYP staining, LI 90%, original magnification 400x.*
Cr-A, chromogranin-A. MAP-2, microtubule associated protein-2. SYP, synaptophysin.
LI, labelling index.

Table III. *Immunohistochemical expression of chromogranin-A (Cr-A), microtubule-associated protein-2 (MAP-2) and synaptophysin (SYP) in different subgroups of patients.*

	Metastatic samples	Non-metastatic samples	Tumour size < 2 cm	Tumour size ≥ 2 cm
Cr-A (mean LI)	(38.6%)	(58.4%)	(53.9%)	(42%)
+	36%	7.6%	21.4%	20%
++	27%	53.8%	28.5%	60%
+++	36%	38%	50%	20%
MAP-2 (mean LI)	(54.4%)	(53.8%)	(55.7%)	(51.1%)
-	10%		7.14%	-
+	20%	30%	28.5%	22%
++	10%	23%	21.4%	11%
+++	60%	46%	42.8%	66%
SYP (mean LI)	(68%)	(84.6%)	(75%)	(82.2%)
-	10%	-	7.14%	-
+	30%	23%	21.4%	33%
++	20%	46%	28.5%	44%
+++	30%	30.7%	42%	22%

mostly low in 42% of the samples in Cr-A analysis, whereas MAP-2 intensities were predominantly strong in 52.2% of the samples. The intensities for SYP did not indicate any particular intensity pattern. The statistical analysis revealed that the intensity of SYP correlated with a head and neck location of the primary tumour, $p<0.02$.

Two of the samples were completely negative for the two tested antibodies; one was negative for MAP-2 and the other for SYP. Both these patients developed metastatic dissemination during the course of the disease. One tumour sample showed high expression of both MAP-2 and SYP simultaneously with low Cr-A expression. This patient presented with disseminated disease and died 8 days after hospitalisation. Variability of the immunohistochemical profile has previously been shown in MCC by Rossiello *et al.* (28).

The present study demonstrates that tumours with stronger expression of neuroendocrine markers, *i.e.* higher neuroendocrine differentiation, have a less aggressive behaviour. This was especially obvious for the Cr-A and SYP expression. The mean LI for Cr-A and SYP in tumours with less aggressive behaviour was 58% and 85%, respectively, compared to those patients who developed metastases with Cr-A 38.6% and SYP 68% respectively.

Staining intensity also seemed have a similar impact on survival. When analysing the moderate (++) and strong (+++) intensity samples together, the difference was even more remarkable. In the Cr-A group, the moderate and strong intensity in samples that expanded to metastases was 63% compared to non-metastatic samples where the percentage was 91%. In the SYP staining the same analysis resulted in 50% and 70% positivity, respectively. Interestingly the MAP-2 expression was quite constant in every subgroup. Tumours with low intensity Cr-A expression metastasised often, but the difference was statistically insignificant ($p=0.08$). Furthermore, low intensities of Cr-A correlated with tumour's invasion to the underlying subcutaneous tissue, ($p<0.09$) and with tumour recurrence ($p=0.07$). All three are important aspects of tumour's progression.

The current results reveal that in small-sized tumours (<2 cm), the Cr-A expression is stronger as are the staining intensities (mean LI 54% vs. 42% in large tumours). This is in contrast to the SYP expression, which was stronger (mean LI 82%) in large tumours (≥2 cm) compared to small tumours (mean LI 75%).

Elevated levels of circulating Cr-A have been detected in the blood of patients with neuroendocrine tumours (29-31). It seems that those patients bearing well-differentiated neuroendocrine tumours had more frequently elevated Cr-A than patients with poorly-differentiated tumours (31). Recently Ferrero and co-workers showed that circulating Cr-A inhibits tumour necrosis factor alpha (TNF) -induced VE-cadherin down-regulation and barrier alteration of cultured endothelial cells. Vascular endothelial growth factor was partially inhibited by Cr-A in in vitro permeability assays. They suggest that circulating Cr-A could help regulate the endothelial barrier function and protect vessels against TNF-induced plasma leakage in pathological conditions characterized by increased production of TNF and Cr-A, such as cancer (32). In general, higher Cr-A expression evaluated by immunohistochemistry from tumour tissue or from blood, seems to indicate tumours with benign behaviour.

In conclusion, MCC shows a high degree of neuroendocrine differentiation that is more evident in less aggressive tumours and in small sized-tumours. The expression of Cr-A and SYP in primary MCC seems to be associated with benign tumour behaviour. Of the immunohistochemical antibodies tested in this study, Cr-A appears to be the most important one in defining the individual course of disease. According to current data, it seems that the value of MAP-2 expression is limited only to confirming the diagnosis of MCC. Further studies have to be performed to establish whether these findings could be applied at the clinical level to select patients for oncological treatments.

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