

Co-Expression of KIT Receptor and Its Ligand Stem Cell Factor in Merkel Cell Carcinoma

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Key Words

Merkel cell carcinoma • KIT • Stem cell factor • Autocrine • Proliferation • Apoptosis

Abstract

Background/Aims: KIT receptor has been implicated in the pathogenesis of cancer, either by mutation or autocrine activation. Merkel cell carcinoma (MCC) is a rare KIT-positive cutaneous tumor. We investigated the co-expression of KIT and its ligand stem cell factor (SCF) in MCC. **Methods:** Sixteen specimens from 13 MCC patients of various tumor stages were examined by immunohistochemistry for SCF, KIT, Ki67/MIB-1 and cleaved caspase 3 expression, and for apoptosis by TUNEL. **Results:** KIT was expressed in 13 of 16 tumors, and SCF in 15 of 16 specimens. Co-expression of KIT and SCF was detected in 12 of 16 tumors. KIT and SCF immunoreactivity scores were independent of tumor stage. Ki67/MIB-1 proliferation rates were high, whereas apoptosis rates were low, and did not depend on KIT or SCF expression. **Conclusion:** Co-expression of KIT and SCF in a high percentage of MCC tumors hints to an autocrine mechanism. KIT and SCF expression in primary tumors and in metastases suggests an early event in Merkel cell transformation.

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Introduction

Merkel cell carcinoma (MCC) or primary neuroendocrine carcinoma of the skin is a rare and aggressive tumor with a substantial incidence rise during the last decades [1–3]. Median survival of MCC in the stage of distant disease is less than 1 year, and despite aggressive chemotherapy subsequent relapses are common and often fatal [4–6]. Novel therapies in oncology are increasingly focusing on blocking cellular receptors that stimulate growth or inhibit tumor cell apoptosis. Such paradigms are the ErbB-2 (HER2/neu) inhibitor herceptin in the treatment of breast carcinoma, the broad-spectrum tyrosine kinase and vascular endothelial growth-factor receptor inhibitor sunitinib in therapy for renal cancer and imatinib, which inhibits BCR/ABL in chronic myelogenous leukemia and KIT in gastrointestinal stromal tumors [7].

The proto-oncogene *KIT* (formerly *c-kit*) was identified in 1987 [8] as the cellular counterpart of the oncogene *v-kit*, the transforming gene in the Hardy-Zuckerman 4 feline sarcoma virus [9]. KIT (CD117) is a transmembrane protein of the receptor tyrosine kinase family that is important for hematopoiesis, gametogenesis, melanogenesis and the development of interstitial cells of Cajal [10]. Following the binding of its ligand stem cell

Table 1. Characteristics of SCF and KIT expression and immunohistochemical findings of proliferation and apoptosis in MCC

Patient No.	Origin of tumor	SCF IRS (PP × SI)	Staining pattern	KIT IRS (PP × SI)	Staining pattern	MIB-1/Ki67 index, %	TUNEL, % positive cells	c-csp 3, % positive cells
1	primary	4 (2 × 2)	C	0	NA	10	1	1
2	primary	2 (2 × 1)	C	8 (4 × 2)	C	ND	ND	ND
3	primary	4 (2 × 2)	C	8 (4 × 2)	C	ND	ND	ND
4	primary	12 (4 × 3)	C, M	2 (2 × 1)	C	35	2	0
4 PR	local relapse	12 (4 × 3)	C	8 (4 × 2)	C	38	0.5	0
5	primary	4 (4 × 1)	C, M	12 (4 × 3)	C	ND	ND	ND
5 PR	metastasis	12 (4 × 3)	C, M	12 (4 × 3)	C, M	55	0.5	0
6	primary	8 (4 × 2)	C	8 (4 × 2)	C	75	1	1
6 PR	metastasis	4 (2 × 2)	C	8 (4 × 2)	C	50	1	1
7	local relapse	4 (2 × 2)	C, M	0	NA	30	1	1
8	local relapse	12 (4 × 3)	C, M	12 (4 × 3)	C	78	2	0
9	local relapse	0	NA	3 (3 × 1)	C	ND	ND	ND
10	local relapse	4 (2 × 2)	C	12 (4 × 3)	C	57	0.5	0
11	metastasis	2 (1 × 2)	C	8 (4 × 2)	C	ND	ND	ND
12	metastasis	2 (2 × 1)	C	8 (4 × 2)	C	70	5	5
13	metastasis	8 (4 × 2)	C	0	NA	90	7	7

Distribution of SCF- or KIT-positive cells in tumor was diffuse, except for cases 6 PR, 10 and 11 where distribution of SCF-positive cells was focal. Details of the scoring systems used for PP and SI are given under Evaluation of Immunohistochemistry in the Patients and Methods section of the main text.

C = Cytoplasmic; c-csp 3 = cleaved caspase 3; M = membrane; NA = not applicable; ND = not done; PR = disease progressed in patient.

factor (SCF, also called the KIT ligand, mast cell growth factor or *steel* factor), KIT undergoes dimerization and becomes activated [11]. Thereafter, receptor autophosphorylation in several tyrosine residues leads to subsequent activation of a variety of signal transduction molecules, including mitogen-activated protein kinases, PI3 kinase or protein kinase C [12]. Several modes of KIT activation have been found in human cancer, including autocrine activation by the co-expression of KIT and SCF reported in small cell lung carcinoma and colorectal cancer [13, 14], and activating mutations of the KIT receptor reported in mastocytosis and gastrointestinal stromal tumors [15, 16].

KIT protein expression has been recently reported in MCC but has been excluded in normal Merkel cells of the skin [17, 18]. The expression of additional growth factor receptors in neoplasms may offer these cells a growth advantage over normal tissues. However, MCC does not contain activating mutations in hot spot regions of the *KIT* gene [19], so the possible pathogenic role of KIT in MCC remains unclear. In the present study, we examined the co-expression of KIT and its ligand SCF in MCC in order to identify possible indications for an autocrine mechanism. We therefore investigated KIT and SCF pro-

tein expression in MCC specimens by immunohistochemistry and studied their correlation with tumor stage and markers of proliferation and apoptosis.

Patients and Methods

Reagents

A rabbit polyclonal anti-human antibody raised against KIT (dilution 1:50; clone A 4502, Dako Corp., Carpinteria, Calif., USA), a mouse monoclonal antibody raised against SCF (dilution 1:200; clone G-3, Santa Cruz Biotechnology, Santa Cruz, Calif., USA), a rabbit monoclonal antibody to cleaved caspase 3 (dilution 1:200; Cell Signaling Technologies, Beverly, Mass., USA) and a mouse monoclonal antibody against Ki67 antigen (dilution 1:50; MIB-1 clone, DakoCytomation, Glostrup, Denmark) were used. The Ultravision LP Detection System with AP Polymer and Fast Red chromogen for visualization of primary antibodies was from Labvision Corp. (Fremont, Calif., USA). The In Situ Cell Death Detection Kit AP was obtained from Roche Applied Science (Mannheim, Germany).

Patients and Tumor Specimens

The study was performed on 4- μ m-thick sections of formalin-fixed, paraffin-embedded archival tissues derived from 13 MCC patients with primary tumor (stage I), cutaneous local relapse (stage II) or distant cutaneous metastatic disease (stage III; table 1) [20]. Additional tumor specimens were available from 3

of these patients, over the course of disease progression (table 1). Institutional review board approval was obtained for the study. Of the 13 patients, 9 were men and 4 were women, and the mean age was 69 years (range 33–83 years). No evidence of neuroendocrine carcinoma of the lung or gastrointestinal tract was found in any of the patients. In all cases, the diagnostic procedure included routine histological examination with HE staining, and immunohistochemistry for typical dot-like immunoreactivity with antibodies directed against cytokeratins and for neuroendocrine markers, such as neuron-specific enolase, synaptophysin, chromogranin and neurofilaments.

Immunohistochemistry for KIT, SCF, Ki67/MIB-1 and Cleaved Caspase 3

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded archival tissues. For the detection of KIT expression, deparaffinized and rehydrated sections were pretreated by microwaving in 10 mM citrate buffer (pH 6.0) for 10 min at 600 W, for epitope antigen retrieval, followed by a 20-min cool-down period. For SCF and cleaved caspase 3, pretreatment was similar to that detailed above, but in a 0.01 M ethylenediamine tetraacetic acid solution (pH 8.0). No antigen retrieval was used for Ki67/MIB-1 stain. After incubation for 2 h with the primary antibodies at room temperature, the Ultravision LP Detection System with AP Polymer and Fast Red chromogen was used according to the manufacturer's instructions for visualization of antibody binding. The epidermis served as an internal positive control for SCF and KIT staining, as keratinocytes stained positive for SCF and melanocytes were positive for KIT. As further positive controls, sections of normal breast epithelium were used for KIT immunoreactivity and normal spleen was used for SCF. Sections of formalin-fixed, paraffin-embedded normal human lymph nodes with known expression of cleaved caspase 3 in germinal centers and melanoma tumor sections with known Ki67/MIB-1 expression were used as positive controls for these markers. Negative controls were prepared using normal mouse IgG or normal rabbit serum in place of the first antibody.

Evaluation of Immunohistochemistry

For each tumor sample, staining intensity (SI) and percentage of cells positive (PP) for KIT and SCF were semiquantitatively evaluated by consensus by 2 investigators (K.K. and E.N.S.) with good overall concordance using the grading system described by Remmele and Stegner [21]. The investigators graded SI on a 4-point scale, where 0 = negative, 1 = weak, 2 = moderate and 3 = strong. The PP was graded on a 5-point scale, where 0 = negative, 1 = <10%, 2 = 10–50%, 3 = 51–80% and 4 = >80%. The immunoreactivity score (IRS) was applied as reported in each tumor as $IRS = PP \times SI$. Only tumor cells with an intense cell membrane and/or cytoplasmic staining pattern (diffuse or dot-like) were evaluated as positive. Very faint or nuclear staining was not considered positive. Also, the distribution (focal or diffuse) of KIT- or SCF-positive cells in the tumor was assessed in each case. PP for cytoplasmic, cleaved caspase 3 and Ki67/MIB-1-positive nuclei in the tumor specimens was assessed by counting at least 100 cells in each of 3 random fields. SI for these antigens was not included in the analysis.

Evaluation of Apoptosis by TUNEL Staining

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end) staining was performed on deparaffinized and

rehydrated tissue sections. Following treatment of sections with proteinase K, DNA strand breaks generated during cellular apoptosis were labeled with fluorescein-conjugated nucleotides to free 3'-OH DNA ends by terminal deoxynucleotidyl transferase enzyme, using the In Situ Cell Death Detection kit AP, according to the manufacturer's instructions. An anti-fluorescein antibody, Fab fragment from sheep, conjugated with alkaline phosphatase (AP) was applied to the samples and after substrate reaction with Fast Red as chromogen, stained cells were analyzed using a light microscope. Sections of formalin-fixed, paraffin-embedded normal human lymph nodes with known apoptosis rates in germinal centers were used as positive controls. PP was assessed by counting at least 100 cells in 3 random fields.

Statistical Analysis

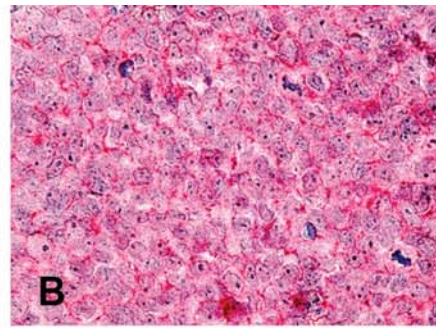
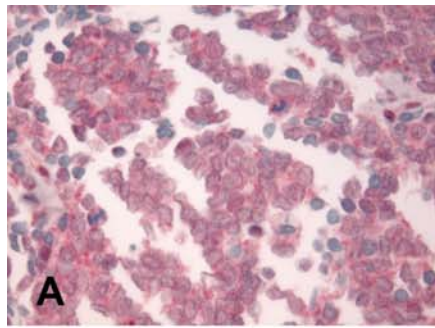
Bivariate analysis using the Spearman's rho correlation coefficient was used to assess the correlation between KIT and SCF expression. KIT or SCF differences in Merkel cell tumors from various stages [stage I (primary tumor), stage II (local relapse) or stage III (distant metastasis)] were evaluated by nonparametric analysis (Kruskal-Wallis). One-way ANOVA was used to compare the means of the Ki67/MIB-1 index between Merkel cell tumors from various stages. Analyses were carried out using SPSS software for Windows from SPSS Inc. (Chicago, Ill., USA). $p < 0.05$ was considered to be statistically significant.

Results

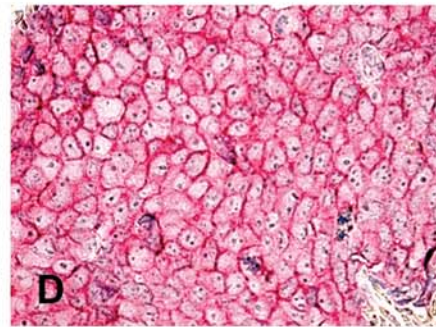
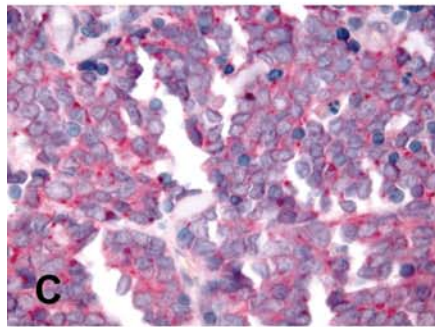
Sixteen lesions from 13 MCC patients were studied using immunohistochemistry to detect KIT, SCF, Ki67/MIB-1 and cleaved caspase 3, and TUNEL was used to detect apoptotic DNA strand breaks. Six of the specimens derived from primary tumors, 5 originated from cutaneous local relapses and 5 were from distant cutaneous metastases.

Stem Cell Factor Expression

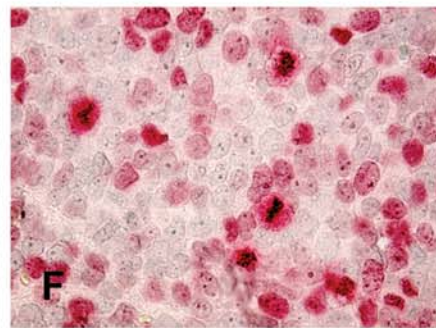
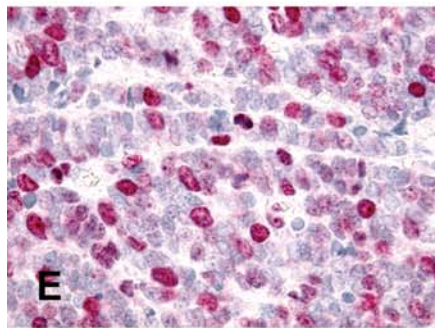
All MCC specimens except 1 stained positive for SCF (table 1). The SCF staining pattern was in most cases cytoplasmic, either diffuse (fig. 1A) or, rarely, dot-like. In a few cases, additional membrane staining was seen, probably corresponding to the membrane-bound form of SCF (fig. 1B). The distribution of SCF-positive cells in most tumors was diffuse, although in 3 it was focal (table 1). Seven of 16 tumors showed widespread positivity for SCF (PP score 4, >80% of cells were positive), whereas only in case 11 were there fewer than 10% of tumor cells positive for SCF (PP score 1). Also the SI, which represents the level of SCF expression in individual tumor cells, could be rated as strong in 4 (SI score 3) and moderate in 8 tumors (SI score 2), whereas in only 3 specimens did tumor cells express SCF weakly (SI score 1). A statistically significant correlation was found between SCF PP and SI



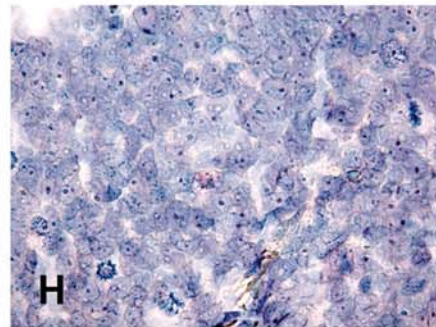
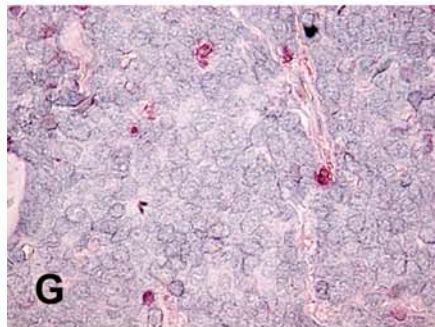
SCF



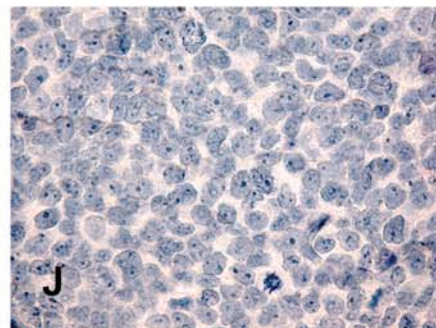
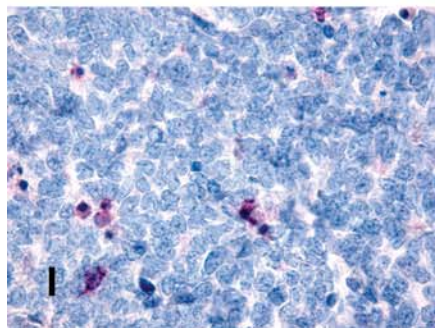
KIT



Ki67/MIB-1



TUNEL



C-CSP3

scores ($r = 0.58$, Spearman's rho test, $p = 0.05$). SCF staining of surrounding tissues was confined to endothelial cells, smooth muscle cells and sweat glands, in most cases at the same intensity as the tumor cells. No free immunoreactive SCF was detected in the dermis.

KIT Receptor Expression

Expression of KIT could be demonstrated in 13 of 16 tumors (table 1). The KIT staining pattern was diffuse cytoplasmic in all positive cases (fig. 1C). One tumor showed both membrane and cytoplasmic staining (case 5 PR; fig. 1D). In all cases, the distribution of KIT-positive cells in the tumor was diffuse. Prevalent expression of KIT (PP score 4, >80% of cells positive) was found in the majority of the positive tumors (11 of 13). The SI of KIT expression by individual tumor cells was rated as strong in 4 cases (SI score 3), moderate in 7 (SI score 2) and weak in another 2. A statistically significant correlation was found between KIT PP and SI scores ($r = 0.86$, Spearman's rho test, $p = 0.01$).

Co-expression of SCF and KIT and Relation to Tumor Stage

Co-expression of SCF and KIT was noted in 12 MCC tumors. To examine whether SCF levels correlated with KIT expression, we compared the PP, SI and IRS score of SCF with the respective parameters of KIT. Bivariate analysis showed that levels of SCF did not influence KIT ($p > 0.05$). SCF and KIT were expressed at early stages of MCC. We investigated whether SCF or KIT expression changes with tumor progression. Therefore, the IRSs for SCF and KIT were examined in relation to the origin of the tumors (primary, local relapse, distant metastasis). Both SCF and KIT IRS did not differ in tumors from the various stages ($p > 0.05$ for SCF and KIT). Also, in cases where primary tumors could be compared either with local relapses or with distant metastases (cases 4, 5 and 6), no tendency for higher expression of either SCF or KIT was found during disease progression. These findings

Fig. 1. SCF (A, B), KIT (C, D), Ki67/MIB-1 (E, F), cleaved caspase 3 (I, J) immunohistochemistry and TUNEL (G, H) with Fast Red as chromogen, from MCC cases 12 and 5 PR, respectively. Note the cytoplasmic staining of tumor cells for SCF (A) and KIT (C) in case 12, and the cytoplasmic/membrane staining of tumor cells for SCF (B) and KIT (D) in case 5 PR. The proliferation marker Ki67/MIB-1 was highly expressed in both cases, but the apoptosis markers TUNEL and cleaved caspase 3 (c-csp 3) were hardly detected in case 5 PR (H, J), and in a minority of tumor cells in case 12 (G, I). Original magnification $\times 400$.

suggest that SCF and KIT expression occur early in disease, and do not depend on tumor stage.

Correlation of SCF and KIT with Proliferation and Apoptosis

In most of the tumors, proliferative activity as determined by Ki67/MIB-1 antibody staining was high (fig. 1E, F; table 1). In 7 of the 11 tumors studied, at least 50% of the tumor cells were Ki67/MIB-1 positive. The Ki67/MIB-1 labeling index did not correlate with SCF or KIT IRS values ($p > 0.05$). In advanced tumors, the mean (\pm SD) Ki67/MIB-1 index tended to be higher (primary 40 ± 33 , local relapse 51 ± 21 , metastasis 66 ± 18), but this was not statistically significant ($p > 0.05$). Apoptosis rates were low in the majority of the MCC biopsies (fig. 1H, J). TUNEL and cleaved caspase 3 were notably positive in only 2 metastases, whereas in all other investigated specimens tumor cells were either negative or only positive in up to 2% of instances (table 1). The 2 tumors with higher apoptosis rates, case 12 with 5% (fig. 1G, I) and case 13 with 7%, had variable SCF and KIT expression.

Discussion

MCC is a rare tumor, but due to its aggressive growth and high malignancy it represents an important tumor model and a challenge in oncology. The molecular biology of MCC is still not fully understood and studies of large tumor panels are rare. In a present study, we found co-expression of KIT receptor and its ligand, SCF, in the majority of MCC tumors. SCF/KIT co-expression may be considered as an indication for the active role of KIT in the pathogenesis of MCC. Ligand-dependent stimulation of KIT by SCF may induce autocrine activation of the receptor, and thus stimulate various cellular activities. Co-expression of SCF and KIT has been reported in breast tumor cells, small cell lung cancer cells and gynecological tumors [22–24]. The percentage of tumors that co-express KIT ligand and receptor in MCC (75%) approximates the ratios reported for nasopharyngeal carcinomas (68%) and gastrointestinal stromal tumors (93%) [25, 26]. The significance of our findings is further strengthened by the fact that hot spot regions of the *KIT* gene in MCC do not contain any activating mutations [19]. This suggests that the autocrine stimulation of KIT by SCF may represent a major pathway of KIT activation in this tumor. During preparation of this paper, a recent publication reported co-expression of KIT and SCF in 16% of MCC tumors [27]. That rate is substantially lower than the one seen in

the present study. However, the low co-expression rate of KIT and SCF in that report may be attributed, at least partially, to the substantially lower KIT positivity, as compared to several other previous publications [17, 28–30]. Differences in positivity rates may result from different antibodies and dilutions, antigen retrieval methods or other variations in staining protocols. Nevertheless, due to the rare co-expression of KIT and SCF in MCC in their report and the SCF staining of surrounding connective tissues, Kartha and Sundram [27] rather favor a paracrine mode for KIT activation. In our study, we have also detected SCF in endothelial and smooth muscle cells as well as in sweat glands, but we found no free SCF in the dermis. Paracrine SCF produced by these cells is more likely to have a secondary role in the activation of KIT, compared to the abundant tumor-derived autocrine SCF in positive tumors. The report by Fenig et al. [31], that the tyrosine kinase inhibitor imatinib mesylate used for the treatment of KIT-positive gastrointestinal stromal tumors inhibited growth of MCC cells in vitro, is in line with our hypothesis that KIT is mainly endogenously activated in MCC.

We further investigated whether SCF or KIT expression changes with tumor progression. Our findings show that SCF and KIT expression occur early in primary disease, and do not depend on tumor stage. This is in agreement with the observation of Su et al. [17], who reported that KIT immunoreactivity does not correlate with the aggressive behavior of MCC. Moreover, we addressed the relation of SCF and KIT expression with proliferation and apoptosis by determining the Ki67/MIB-1 proliferation index and the TUNEL and cleaved caspase 3 apoptosis indices in MCC tumors. SCF, besides its growth-stimulatory effects, has been reported to rescue cells from caspase 3-dependent apoptosis induced by various death signals [32, 33]. In our study, the Ki67/MIB-1 proliferation index was in general high and the apoptosis rate was found to be low in the MCC tumors. The absence of any clear correlation with SCF or KIT expression may be attributed to the primary involvement of the SCF/KIT system in the oncogenesis of MCC, with its expression to remain unaltered during disease progression. Similarly, Feinmesser et al. [28] suggested that KIT expression is a relatively early event in primary transformation of MCC which persists in metastases, and is not a marker for tumor progression. Regarding apoptosis, Lombart et al. [29] found no correlation between expression of KIT and various pro- and anti-apoptotic markers, such as p53 and bcl-2. In addition, MCC is an aggressive tumor that is characterized by high homogeneously distributed proliferation with low and grossly invariable apoptosis [34]. So

far, high proliferation and low apoptosis in the tumor have been consistently associated with a bad prognosis [35, 36]. Moreover, the biochemical events that regulate the expression of cytokine receptors in the presence of their ligands are complex. SCF binding to KIT receptor accelerates the turnover of KIT by inducing internalization of receptor-ligand complexes, followed by polyubiquitination and degradation of KIT [37]. The few MCC tumors that completely lacked either SCF or KIT may have acquired other changes that enhance proliferation or block apoptosis.

In summary, the present study demonstrated the co-expression of KIT and its ligand SCF in 75% of MCC, suggesting an important function of this receptor/ligand signaling system in this tumor. In several other types of cancer, the simultaneous expression of both cytokine and the corresponding receptor from the cells is a typical indication for an autocrine mechanism. Early activation of the SCF/KIT system seems to occur in MCC, since KIT and SCF expression were not related to tumor stage. However, the exact function of ligand-mediated activation of KIT in MCC has to be clarified in further studies. The potential use of KIT kinase inhibitor-based therapies, as already applied in other tumors, should be also considered in MCC [38]. Support for this strategy is provided by the observation that imatinib inhibits growth of MCC cells in vitro [31]. Imatinib has been also considered for blocking endogenous platelet-derived growth factor-mediated activation of fibroblasts in scleroderma [39]. However, such therapeutic concepts for cancer have to be further investigated, since several molecular mechanisms are responsible for primary and secondary imatinib resistance in tumors [40].

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