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Huntingtin Interacting Protein 1: a Merkel Cell Carcinoma Marker That Interacts with c-Kit

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Abstract

Merkel Cell Carcinoma (MCC) is a neoplasm thought to originate from the neuroendocrine Merkel cells of the skin. While the prevalence of MCC has been increasing, treatments for this disease remain limited due to a paucity of information regarding MCC biology. We have found that the endocytic oncoprotein Huntingtin interacting protein 1 (HIP1) is expressed at high levels in close to 90% of MCC tumors and serves as a more reliable histological cytoplasmic stain than the gold standard, cytokeratin 20 (CK20). Furthermore, high anti-HIP1 antibody reactivity in the sera of a cohort of MCC patients predicts the presence of metastases. Another protein that is frequently expressed at high levels in MCC tumors is the stem cell factor (SCF) receptor tyrosine kinase, c-Kit. In working towards an understanding of how HIP1 might contribute to MCC tumorigenesis, we have discovered that HIP1 interacts with SCF activated c-Kit. These data not only identify HIP1 as a molecular marker for management of MCC patients but also show that HIP1 interacts with and slows the degradation of c-Kit.

Keywords

Merkel Cell Carcinoma; HIP1; Autoantibodies; Receptor Tyrosine Kinase; endocytosis

Introduction

Merkel Cell Carcinoma (MCC) is an aggressive skin cancer thought to be derived from the sensory Merkel cells of the skin (Bichakjian *et al.*, 2007). Though the overall incidence of this disease is low and affects approximately 1400 patients per year in the United States, this cancer is associated with a poor prognosis, and most patients with metastatic disease do not survive more than 5 years (Bichakjian *et al.*, 2007). Clinical management of patients with MCC is limited due to a lack of prognostic markers and effective therapies. These

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limitations stem from a lack of understanding of the biology of MCC's initiation, maintenance and progression to the metastatic stage. Currently, surgical excision of the primary tumor and radiation remain the main therapeutics for MCC (Bichakjian *et al.*, 2007).

Though receptor tyrosine kinases (RTKs), such as c-Kit, have been shown to be increased in expression in MCC, convincing clinical data regarding the effectiveness of specific c-Kit inhibitors such as imatinib, on patient survival are not yet available (Lemos and Nghiem, 2007). Recently, a virus designated Merkel Cell Polyoma Virus (MCV) has been found in tumors from MCC patients but not their normal skin tissue (Feng *et al.*, 2008). In contrast, its use as a serum biomarker is not clear due to limited specificity. Antibodies against the virus have been found in a large number of tumor-free individuals as well (Carter *et al.*, 2009). Furthermore, its role in initiating MCC remains hypothetical (Gandhi *et al.*, 2009). A better understanding of how c-Kit or MCV contribute to the induction, maintenance and progression of MCC will facilitate the development of effective therapies.

Huntingtin interacting protein 1 (HIP1) is a highly conserved protein that interacts with components of the endocytic machinery, including 3-phosphoinositides, clathrin and AP-2 (Engqvist-Goldstein *et al.*, 1999; Engqvist-Goldstein *et al.*, 2001; Hyun and Ross, 2004; Metzler *et al.*, 2001; Mishra *et al.*, 2001; Rao *et al.*, 2001; Waelter *et al.*, 2001). All of these molecules are involved in the clathrin-mediated internalization of surface receptors. Unlike AP-2 (Mitsunari *et al.*, 2005), HIP1 is not necessary for embryogenesis or early post natal development, but young adult mice deficient for HIP1 do develop a degenerative phenotype (Oravecz-Wilson *et al.*, 2004). In addition, HIP1 over-expression transforms fibroblasts (Rao *et al.*, 2003b) and prostate epithelial cells (Wang *et al.*, 2008) and HIP1 transgenic mice develop plasma cell neoplasms (Bradley *et al.*, 2007b).

In addition to its transforming activity, high HIP1 expression is associated with a variety of human cancers, including prostate, colon, breast, brain, and lymphoid cancers (Bradley *et al.*, 2007a; Bradley *et al.*, 2007b; Rao *et al.*, 2003b; Rao *et al.*, 2002). HIP1 expression in prostate tumors is associated with a poor prognosis, suggesting that HIP1 over expression may functionally promote tumorigenesis (Bradley *et al.*, 2005). Anti-HIP1 antibodies have been detected in the sera of patients with prostate, lymphoid, and brain cancers more frequently than in the sera of cancer-free individuals (Bradley *et al.*, 2007a; Bradley *et al.*, 2005; Bradley *et al.*, 2007b). These findings indicate that testing for anti-HIP1 antibodies in serum may serve as a useful non-invasive test to detect the presence, recurrence or progression of some human tumors.

Further investigation into the role of HIP1 in tumorigenesis has demonstrated an association between the presence of this oncoprotein and enhanced RTK expression (Bradley *et al.*, 2007a; Rao *et al.*, 2003b). These findings are not unexpected due to the association of HIP1 with components of the clathrin-mediated endocytosis pathway, which is a mechanism for modulation of receptor levels. Indeed, not only is HIP1 over expression in tumors associated with the over expression of RTKs, but HIP1-mediated transformation can be blocked with tyrosine kinase inhibitors (Rao *et al.*, 2003a). We have found that co-expression of HIP1 with the EGFR increases the half life of the EGFR upon EGF stimulation (Hyun *et al.*,

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2004) and that HIP1 physically associates with the EGFR (Bradley *et al.*, 2007a). Others have found that HIP1 also stabilizes and associates with the fibroblast growth factor receptor 4 (FGFR4) (Wang *et al.*, 2008). These interactions together with HIP1's over-expression in multiple cancers suggest that HIP1-mediated transformation may occur via concomitant increases in multiple RTK signals.

The potential of HIP1 as a clinical marker for several types of tumors, along with its high expression in neural crest derived peripheral neurons (Rao *et al.*, 2002) led us to examine the possibility that HIP1 could serve as a marker for MCC. We evaluated a large series of MCC tissue samples and found vastly elevated HIP1 protein levels compared to normal surrounding skin tissue. We also detected high levels of anti-HIP1 antibodies in sera from a separate cohort of MCC patients. Some of the patients with metastatic MCC exhibited higher levels of anti-HIP1 antibodies compared to MCC patients with localized disease. Furthermore, in an effort to discover how HIP1 expression may functionally contribute to MCC biology, we discovered that HIP1 physically associates with and stabilizes c-Kit, a RTK specifically expressed at high levels in MCC (Sattler and Salgia, 2004; Su *et al.*, 2002).

Results

MCC specimens demonstrate high levels of HIP1 staining

To evaluate HIP1 as a possible MCC marker, paraffin-embedded tissue samples from MCC tumors and the similar “round blue cell” neuroendocrine tumor, small cell lung cancer (SCLC), were immunostained for HIP1 expression (Fig. 1*A*). These tumors were evaluated for both HIP1 expression level and cellular localization. HIP1 is not expressed at high levels in normal skin with the exception of vascular endothelium (Rao *et al.*, 2002). Tumor tissue was visually scored for HIP1 expression on a scale of 0–3, in which a score of 3 represented the highest HIP1 staining and a score of zero indicated a lack of staining (Bradley *et al.*, 2007b).

Eighty nine percent (n=25/28; 89%) of MCC tissue samples in our first MCC patient cohort exhibited high HIP1 expression (Table I). MCC tissue samples displayed both diffuse cytoplasmic staining as well as perinuclear dot patterns of staining. HIP1 expression occurred much more frequently in MCC tumors than in SCLC tumors (n=5/12 for SCLC; 42%). In particular, frequency of HIP1 over-expression was significantly higher in metastatic MCC as compared to metastatic SCLC (Table I). This finding is clinically significant since MCC and SCLC are often difficult to distinguish from one another in the metastatic setting.

Additionally, as one would predict with increased HIP1 protein expression in MCC tumors, using mRNA microarrays and a distinct cohort of patients a 6-fold average increase in HIP1 message was detected in MCC tumors. A cohort of 30 patients was studied and 29 of them displayed an elevated HIP1 message compared to squamous cell carcinomas. High HIP1 levels have not been observed in squamous cell carcinoma (Rao *et al.*, 2002). In contrast to HIP1, HIP1-related, HIP1's only known mammalian relative was not elevated at the message level (personal communication, Paul Harms, University of Michigan).

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We also compared HIP1 tumor staining to the known MCC markers, CK20 and c-Kit (Figure 1*B*). A separate cohort of 14 MCC tumors from archived samples from MCC patients (all of whose diagnoses were based on immunophenotyping and clinicopathological correlations at the University of Michigan) were used for generation of a tissue microarray (TMA) to make HIP1, CK20, c-Kit comparisons. Each tumor was represented by 3 different spots on the TMA slide for purposes of better tumor coverage. As is evident in the top row, HIP1 staining for MCC was very strong, diffuse and sensitive (100% positive; n=14/14). In comparison, CK20 staining was less reliable and positive in only 64% of the tumor samples (row 3 vs. the top row; n=9/14). This frequency of CK20 staining is lower than previous reports where it has been found to be positive in up to 80% of MCC tumors. This also does not reflect the original pathological assessment for each of the tumors from which they were derived, as 13 of the 14 were reported as positive for CK20 staining. This difference is likely due to the use of a TMA rather than the entire slide for testing each tumor. Because CK20 staining is not as uniformly distributed in the tumor cells as HIP1 staining is, it is possible that a positive tumor could test falsely negative for CK20 due to there being less tissue represented on a TMA. These data nevertheless indicate that the chance of misdiagnosing a MCC when staining for CK20 is greater than when staining for HIP1. Because the HIP1 antibody stained every tumor, the HIP1 test was positive in all tumors that tested positive for CK20 (third row vs. top row) and another important MCC marker c-Kit (second row vs. top row).

HIP1 does not affect the development or maintenance of normal Merkel cells

In order to determine whether HIP1 is necessary for the development of normal Merkel cells, we analyzed the skin of wild-type and HIP1-null mice (Oravecz-Wilson *et al.*, 2004). Anti-CK20 antibodies were used to identify mature Merkel cells in the mouse tail skin and vibrissae, locations where Merkel cells generally congregate around hair follicles. No visible changes in the abundance of mature Merkel cells were observed in the HIP1-null mouse skin as compared to wild-type littermate skin (Fig. 1*C*). These data indicate that HIP1 is not required for the development or maintenance of normal Merkel cells.

MCC patients harbor anti-HIP1 auto-antibodies in their blood

To detect the presence of anti-HIP1 antibodies in MCC patient blood, patient sera were tested for immune-reactivity to HIP1 antigens as described previously (Bradley *et al.*, 2005). Initially, serum samples were screened against the previously described C-terminal HIP1 recombinant antigen (Bradley *et al.*, 2007a; Bradley *et al.*, 2005; Bradley *et al.*, 2007b). Eighty-five percent of MCC patients (n=34/40; 85%) displayed the presence of autoantibodies (Supplementary Figs 1A and 2). This frequency was similar to that previously found in patients with glioblastoma multiforme (Bradley *et al.*, 2007a). This high antibody prevalence and the elderly nature of this population (Table II) raised the question of whether the humoral response was the result of co-existence of other types of tumors with elevated HIP1 levels. We found that there were many co-existing basal and squamous cell carcinomas in this patient cohort but very few other tumors. There was no correlation of a humoral response with prior cancer diagnoses (Supplemental Table I). The high degree of HIP1 seropositivity to this antigen also made intergroup comparisons difficult, so, in the interest of improving the specificity of the test, patient sera were also tested for reactivity

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against a different HIP1 recombinant antigen that encoded the amino terminus. This antigen contains the lipid-binding (ANTH), clathrin-binding, and AP2-binding domains (Supplementary Fig 1*C*). Only thirty percent of MCC patients (n=12/40; 30%) harbored antibodies against the HIP1 N-terminal antigen (Supplementary Fig. 1*A* and 1*B*). This was a low enough frequency for possible clinical correlations between patients that were positive or negative for these antibodies to be made (see below).

Sera from patients with metastatic MCC exhibited high antibody reactivity to the HIP1 N-terminus

To examine the possibility that anti-N-terminal HIP1 antibodies in MCC patient sera could correlate with a biological outcome, the humoral response to the N-terminus of HIP1 in MCC patients were compared with a number of clinical parameters. These parameters included tumor size, disease status at time of blood draw, presence of metastasis at time of blood draw, past (or concurrent) other cancer diagnoses (Supplemental Table I), survival 2.5 years after blood draw, age, and gender. Of these parameters, only the presence of metastasis and female gender displayed a significant association with high serum reactivity to HIP1.

Patients with metastatic MCC tested positive for elevated anti-N-terminal HIP1 antibody reactivity much more frequently than patients with localized primary tumors (Table II; Fig. 3; 46% versus 0%; $p < 0.005$; Pearson χ^2). This test in this cohort was 100% specific. No patient with localized disease tested positive for anti-HIP1 antibodies in their serum. Hence, a positive test for anti-N-terminal HIP1 antibody reactivity marked the presence of metastatic disease. Additionally, a non-significant trend was also observed (Table II) between auto-antibody presence and extensive metastatic disease compared to microscopic and local lymph node metastases (Table II). To contrast, the frequent presence of antibodies against the C-terminal HIP1 antigen in patient sera (85% of patients) did not significantly associate with metastasis as 62% of patients with localized disease also tested positive for antibodies against the HIP1 C-terminal antigen.

As might be expected, survival after a positive test for the anti-N-terminal HIP1 antibodies was poor because the patients were of advanced stage (metastatic disease). In total, 5 out of 10 patients (50%) with metastatic disease and high antibody reactivity against the HIP1 N-terminus (Fig. 2; group 1) were deceased from disease after a 2.5 year observation period. This contrasts with the fact that 25% (3/12) of the patients with metastatic disease and negative anti-N-terminal HIP1 antibody tests were dead at 2.5 years (below the line in Figure 2; group 2). This was not a significant survival difference. In contrast to the 25–50% death rate in the patients with metastatic disease was a 100% survival of those patients with localized disease and a low anti-N-terminal HIP1 antibody reactivity (right hand side of Fig. 2; group 3). Two of the original patients in cohorts 1 and 2 were lost to follow-up explaining the decrease in patient numbers between cohorts 1 and 2 in the metastasis (Figure 2) and subsequent 2.5 year survival data described above.

A disproportionate number of female patients had elevated anti-HIP1 antibody reactivity in their blood. Approximately 45% of female patients had high anti-N-terminal HIP1 antibody reactivity, while only 11% of male patients had high anti-N-terminal HIP1 antibody

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reactivity (data not shown). The positive association between anti-HIP1 antibodies and female gender was significant ($p < 0.05$, Pearson χ^2). In fact, when males were excluded, the association between metastasis and the presence of N-terminal antibodies tightened ($p < 0.001$). In addition, no association between the presence of anti-N-terminal HIP1 antibodies and age or tumor size was identified in this elderly patient population (Table II).

HIP1 interacts physically and functionally with the c-Kit RTK

MCC tumors express significant levels of several RTKs (Brunner *et al.*, 2008); however, expression of the receptors previously reported to interact with HIP1, including EGFR (Bradley *et al.*, 2007a) and FGFR4 (Wang *et al.*, 2008), were not among those found to be increased in MCC tissue. Thus, we tested the ability of HIP1 to physically interact with c-Kit, an RTK that is frequently evaluated in these patients because it is expressed at high levels in MCC tumors (Fig. 1*C*) (Brunner *et al.*, 2008). Since c-Kit is rarely expressed in normal adult tissues other than progenitors of the hematopoietic system such as rare hematopoietic stem cells (Bernex *et al.*, 1996), we co-expressed the cDNA for c-Kit along with the cDNA for HIP1 in HEK 293T cells to obtain enough material for analysis. HIP1 was immunoprecipitated from the whole cell lysate using rabbit anti-HIP1 polyclonal antibodies (UM410 or UM323) or pre-immune sera. Western blot analysis of the immunoprecipitate showed that c-Kit was specifically co-immunoprecipitated with HIP1. In the absence of over-expressed HIP1, there was no co-immunoprecipitation (Fig. 3*A*, left hand panel, lane 1 vs. 2). In addition, the reverse immunoprecipitation with antic-Kit antibodies also demonstrated co-immunoprecipitation with HIP1 (Fig. 3A, right hand panel, lane 4 vs. 5).

We hypothesized that if this interaction was functionally related to endocytosis, it may be dependent on activation of the receptor. Addition of SCF, the c-Kit ligand, one hour prior to cell collection for immunoprecipitation did indeed enhance the detected interaction between c-Kit and HIP1 (Fig. 3*A*, lanes 3 and 6). Western blot analysis of whole cell lysates from these cells showed no differences in c-Kit expression in those cells treated with SCF as compared to untreated cells. We also observed an interaction between endogenous HIP1 and c-Kit in a MCC cell line (MCC565) when SCF was added to the cell media 1 hour prior to cell harvest. This interaction was not observed in the absence of SCF (Fig. 3*B*). We also examined whether HIP1 over expression has the ability to inhibit the degradation of the c-Kit receptor similar to the effect of HIP1 on EGFR and FGFR4 levels. Indeed, HIP1 stabilized c-Kit following SCF stimulation of starved and cycloheximide treated cells. The receptor levels were significantly higher one and two hours after stimulation when HIP1 was over expressed with c-Kit (Fig. 3*C*). These data together suggest that the interaction of HIP1 with c-Kit is functionally important.

Discussion

MCC is a rare cancer, for which investigation of the molecular mechanisms of its cause and maintenance, to guide the development of better treatment regimens, has only recently received significant attention. Patients with MCC have a poor prognosis similar to patients with other neuroendocrine tumors, such as SCLC. In contrast to SCLC (Socinski and

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Bogart, 2007), MCC patients suffer from a lack of therapies and prognostic markers (Bichakjian *et al.*, 2007). In this study, we demonstrate not only that HIP1 is a useful immunohistochemical marker for MCC but also that auto-antibodies against the N-terminus of HIP1 in patient sera predict the presence of metastatic disease. The discovery of high HIP1 levels in the tumors of MCC patients has led to the hypothesis that HIP1, which acts as an oncoprotein when expressed at high levels, contributes to the mechanism(s) of MCC development, maintenance or progression. Our data suggest that over expression of HIP1 leads to elevated RTK levels through its prevention of receptor degradation and therefore may increase pro-growth signals leading to transformation of Merkel cells.

The high levels of HIP1 or fragments of HIP1 released from the cytoplasm of necrotic or dying MCC tumor cells likely serve as immunogens in MCC patients, leading to the cancer specificity of a positive anti-HIP1 antibody test (Bradley *et al.*, 2005). Here, we employed both a different amino-terminal HIP1 test antigen and the previously described carboxy-terminal HIP1 test antigen for anti-HIP1 antibody analysis (Bradley *et al.*, 2005) and found that antibodies against the amino-terminus of HIP1 are present more frequently in patients with metastatic MCC than in patients with localized MCC. This association was not found for the carboxy-terminal antigen, as many more patients had a positive test result making correlation with prognostic factors insignificant. Notably, although anti-HIP1-N-terminal antibodies were associated with metastasis, no other clinical parameter, such as tumor size, was associated with antibody test results. Since locally treated MCC is often recurrent and can unpredictably metastasize and become unmanageable (Bichakjian *et al.*, 2007), future prospective studies of this upgraded anti-HIP1 blood test using both antigens will be important to execute. These clinical trials will also help to determine whether the presence of these antibodies serves as a marker of existing metastatic disease alone or whether the antibodies are predictive of tumor metastatic potential. If the presence of HIP1 auto-antibodies can serve as a predictive marker in early disease stages, then positive blood test results could indicate the need for more aggressive early adjuvant therapy.

The prognostic results associated with this improved HIP1 N-terminal antigen blood test support re-evaluation of patients with other metastatic and localized epithelial cancers for antibodies against the N-terminal HIP1 antigen compared to patients with localized disease. For example, since HIP1 over expression in prostate cancer tumors is associated with poor prognosis (Rao *et al.*, 2002), it is possible that relapsing patients will also have increased anti-N-terminal HIP1 antibody reactivity like metastatic MCC patients. Because the decision to surgically resect prostate tumors is often difficult due to potential urologic side effects, the value of a blood test to predict which tumors are likely to metastasize would be useful. Also, a combination test for both HIP1 N-terminal and C-terminal antibodies may be more sensitive and specific than either test alone.

The gender specific association of metastases with the amino-terminal anti-HIP1 antibodies that was found mainly in the female patients was intriguing. This observation is similar to the known increased frequency of autoimmune diseases in women compared to men (Lockshin, 2006). In addition, a recent report found that in MCC patients female gender correlated with better survival (Kaae *et al.*, 2010). Perhaps, the ability to immunologically respond to high HIP1 levels improves prognosis. For example, in B-cell lymphoid

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malignancies anti-HIP1 reactivity correlated with good outcome (Bradley *et al.*, 2007b). Of course, future tests of the prognostic value of antibodies to the HIP1 N-terminal antigen will still include men, since this study examined too few men with metastases (n = 10) to be conclusive. Future prospective trials will be important to either confirm or refute these initial gender specific results.

The mechanisms of how HIP1 transforms cells remain a subject of investigation. The prevailing hypothesis is that HIP1 inhibits the degradation of active RTKs during the process of receptor-mediated endocytosis (Hyun and Ross, 2004) due to the data showing that its over expression stabilizes RTKs following receptor activation (Hyun *et al.*, 2004). For example, cells transformed by HIP1 over-expression have elevated EGFR levels and specific EGFR inhibitors inhibit the transformed phenotype, suggesting that this receptor stabilization is an essential element of HIP1-mediated transformation (Rao *et al.*, 2003a). Prior reports have also shown that HIP1 physically interacts with EGFR (Bradley *et al.*, 2007a) and FGFR4 (Wang *et al.*, 2008). Neither of these receptors is known to be expressed in MCC, suggesting that the tumorigenic function of HIP1 in MCC may be mediated through modulation of a different RTK.

For example, The c-Kit RTK is often over expressed in MCC as well as other tumor types such as breast tumors, SCLC, colorectal cancers, and gastrointestinal stromal tumors, where it is a pharmacological target of imatinib (Sattler and Salgia, 2004; Su *et al.*, 2002). A recent report has linked c-Kit over-expression to poor prognosis in MCC (Andea *et al.*, 2010). The finding here of HIP1's ability to interact physically with c-Kit and to increase c-Kit levels provides a plausible mechanism for how HIP1 might promote tumorigenesis in MCC. It is less clear, though important to determine, how high HIP1 levels in MCC might relate to MCV infection of Merkel cells. If these two abnormalities are mechanistically linked, i.e. if HIP1 is upregulated by MCV or visa versa, then targeting the regulator would be expected to affect the other. This possibility could be tested *in vitro* with knockdown of HIP1 or large T antigen to determine the effect on each other's expression.

In summary, we report that HIP1 is to our knowledge a previously unreported marker of MCC, a neuroendocrine tumor of the skin, and that a blood test for anti-HIP1 antibodies may provide prognostic information. The original assay (Bradley *et al.*, 2005) was supplemented by use of a different recombinant HIP1 N-terminal antigen. The findings with this test will necessitate future studies to determine whether the distinct anti-HIP1 antibodies are reflective of metastatic potential of other tumors (prostate, lymphoid and brain) expressing high levels of HIP1. Prospective trials that include a larger number of patients and serial blood samples will be needed to validate these results to facilitate improved management of MCC patients. Finally, we report that HIP1 physically interacts with and stabilizes the c-Kit RTK and that this interaction is modulated by the c-Kit ligand, SCF. Results of future studies that elucidate all of the RTKs that interact with HIP1 in MCC, the domains through which these interactions are mediated, and the effects of these interactions on transformation and signaling will be enlightening. Designing drugs for specific inhibition of the interactions between HIP1 and RTKs may prove therapeutic to many cancer patients including those afflicted with MCC.

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Materials/Subjects and Methods

MCC and small cell lung cancer (SCLC) tissue samples

Archived formalin-fixed and paraffin-embedded MCC and SCLC tissue samples were obtained from the Pathology Department at the University of Michigan Medical Center. Diagnoses were determined by CK20, thyroid transcription factor-1 (TTF-1), synaptophysin, chromogranin A, morphology and the site of the primary tumor. Tissue microarrays were generated from 14 of the MCC patient tumors as described previously (Perrone *et al.*, 2000) and cores were spotted in triplicate. These patient samples were not linked to clinical data or other identifying information.

Immunohistochemical staining

Immunohistochemical staining for HIP1 was performed as described previously (Bradley *et al.*, 2007a) with appropriate negative (no primary antibody) and positive (glioblastoma) controls. Staining for Merkel cells in mouse skin was performed using the mouse monoclonal antibody Ks20.8 (ThermoScientific). Photomicrographs of the immunohistochemical staining were taken with a model BX41 Olympus microscope.

Patients analyzed for serum antibodies against HIP1

The study of patients with MCC and serum levels of anti-HIP1 antibodies was approved by the University of Michigan Internal Review Board where written and informed patient consent and adherence to the Helsinki Guidelines was confirmed. Serum from 40 clinico-pathologically confirmed MCC patients was collected at the University of Michigan Merkel Cell Carcinoma clinic in a period of 6 months ranging from 2007 to 2008. Serum was aliquoted into 20 μL portions for single use to avoid freeze thaw cycles, and stored at −80 °C. The ages, genders, and tumor stages of these patients are displayed in Table II.

Preparation of HIP1 antigen

Glutathione *S*-transferase (GST) HIP1 (3′ and 5′) fusion cDNAs were used to generate C-terminal and N-terminal recombinant antigens, respectively. The C-terminal antigen has been previously described (Bradley *et al.*, 2005). The N-terminal antigen was generated by sub-cloning an in-frame GST fusion protein to the 5′ end of the region of HIP1 that terminates at the internal EcoRI site in the HIP1 nucleotide sequence. The antigen was produced in bacteria and purified as previously described for the C-terminal antigen (Bradley *et al.*, 2005).

Test for anti-HIP1 antibodies in MCC patient serum

Immunoblot of patient serum was performed as previously described (Bradley *et al.*, 2005). The optical density reflective of serum antibodies bound to the HIP1 antigen was measured using the ImageJ program and measurements were made after subtracting the background density above and below the HIP1 antigen in each lane. Lanes that had an optical density of at least 20% of the internal positive control (JMM, a patient with ALL from reference (Bradley *et al.*, 2007b), was used for the C-terminal antigen and MCC8, a patient with MCC in this cohort for the N-terminal antigen) were considered positive. From prior studies of

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mice and humans with prostate (Bradley *et al.*, 2005) and lymphoid cancers (Bradley *et al.*, 2007b), a cutoff of more than 20% of a strong standard (internal positive control) was used to determine if the sera was negative (<20%) or positive (>20%) for reactivity.

Statistics

Data were analyzed using GraphPad Prism 5 statistical software and Image J densitometry analysis software. Statistical significance values for Table I and Table II were calculated using Pearson χ^2 analysis. Statistical significance values in Figure 3 were calculated using the Student's t test.

Miscellaneous

Immunoprecipitations, Western analysis and RTK stabilization assays were performed as described previously (Hyun *et al.*, 2004) and specific details are also included in supplemental materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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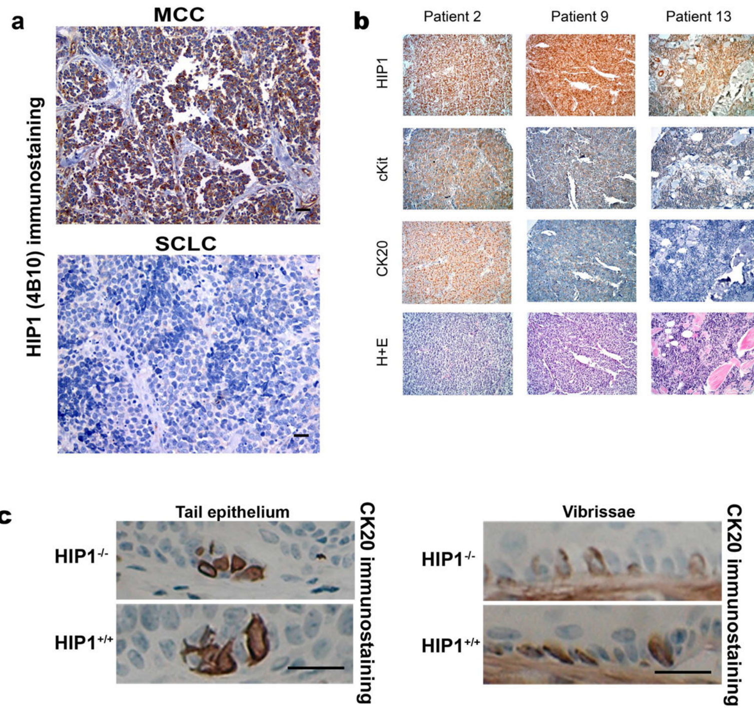

Figure 1. HIP1 is expressed at high levels in primary MCC but not in SCLC and is not required for normal Merkel cells

A, Example of HIP1 staining in MCC compared to SCLC tumors. Scale bar represents 50 μm.

B, Three representative patient tumors co-immunostained for HIP1 (UM4B10), c-Kit, CK20, and Hematoxylin and Eosin (H+E). These tumors were selected from a MCC tissue microarray that contained 42 MCC spots from 14 patients.

C, CK20 staining of Merkel cells in wild-type and *Hip1*$^{null/null}$ (Oravecz-Wilson *et al.*, 2004) mouse tail skin and vibrissae. Scale bars represent 50 μm.

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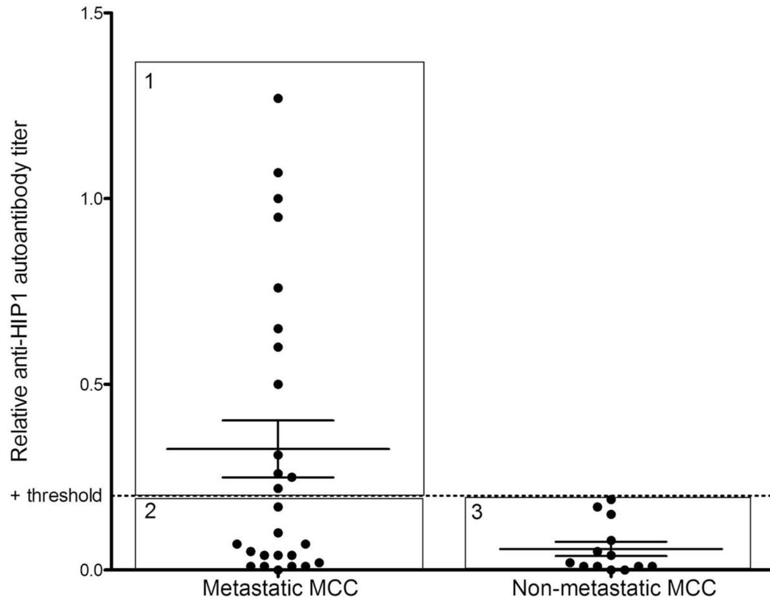

Figure 2. Antibody reactivity against the N-terminal antigen is elevated in metastatic MCC patients

Individual dots represent relative antibody titers from patients with either metastatic MCC or localized MCC. Members of groups 1–3 defined by test result and whether or not their disease was metastatic are enclosed by the boxes. Mean and standard error of the mean for each data set are indicated by horizontal and vertical lines, respectively.

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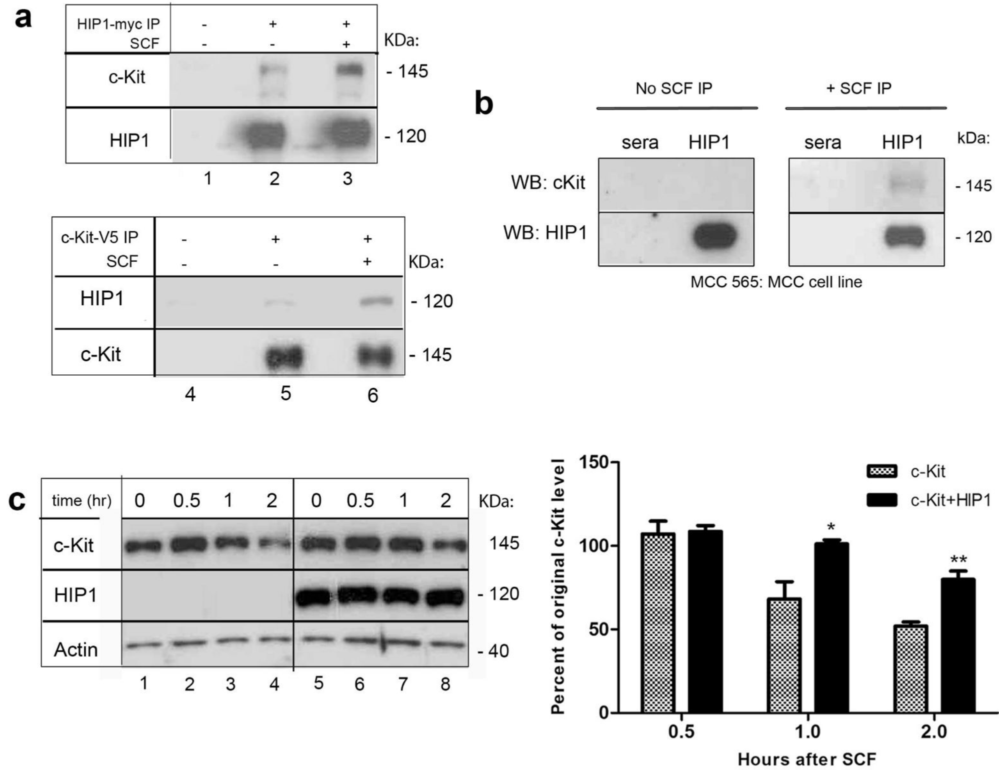

Figure 3. HIP1 interacts with c-Kit a RTK that is expressed at high levels in MCC

A, Association of HIP1 with c-Kit in HEK 293T cells was detected by co-immunoprecipitation. This interaction was enhanced by stimulation with the c-kit ligand SCF (lanes 3 and 6).

B, HIP1 associates with c-Kit in a SCF stimulated MCC cell line. The MCC565 cell line was, or was not treated with SCF for one hour prior to collection. HIP1 was precipitated from the cell lysates (9mg) using the rabbit polyclonal antibody UM410 and blotted for human HIP1 or c-Kit.

C, Prolongation of c-Kit's half life by HIP1 was observed in three independent experiments. On the left is a representative western blot demonstrating the stabilization of c-Kit by HIP1 compared to vector transfected cells after treatment of cells with SCF.

* $p < 0.05$, ** $p < 0.01$, Two-tailed t-test.

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Table I

HIP1 immunostaining of paraffin-fixed MCC and SCLC tissue.

Tumor type	3+	2+	1+	No staining	HIP1 positivity (%)
MCC Total (n=28)	3	8	14	3	89**
Primary	2	4	9	1	94
Metastatic	1	4	5	2	83*
SCLC Total (n=12)	0	1	4	7	42
Primary	0	0	3	2	60
Metastatic	0	1	1	5	29

** Significant difference compared with patients with SCLC ($p < 0.0025$, χ^2 analysis).

* Significant difference compared with patients with metastatic SCLC ($p < 0.025$, χ^2 analysis).

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Table II

Frequency of positive anti-HIP1 (N-terminal) antibody blood test in metastatic MCC.

Patient status	Positive	Negative	Frequency	Age (years) ±SD	Male (%)
All MCC patients (n=40)	12	28[#]	0.33	69 ± 12	45
No metastases (n=13)	0	13	0.00	71 ± 12	54
Metastatic disease (n=26)	12	14	0.46**	67 ± 11	42
Extensive Metastases	6	3	0.67	72 ± 12	33
Local Metastases	3	4	0.38	63 ± 10	50
Micrometastases	3	6	0.33	66 ± 9	44

** Significant difference compared with patients without metastatic disease ($p < 0.005$, χ^2 analysis)

[#] One patient was lost to follow-up and metastatic status could not be determined