Identification of the M541L Sequence Variation of the Transmembrane KIT domain in Merkel Cell Carcinoma

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Abstract. *Background: Merkel cell carcinoma (MCC) is an aggressive KIT-positive cutaneous tumor. KIT mutations are considered to play a key role in the pathogenesis of various neoplasms, but have not been found so far in MCC. The aim of the present study was therefore to investigate the presence of KIT mutations in MCC. Materials and Methods: The entire coding region of KIT in the MCC cell line MCC-1 was sequenced. KIT exon 10 was amplified from archival paraffin-embedded MCC specimens by PCR and sequenced. Results: Exon 10 M541L KIT sequence variation, which confers increased sensitivity to KIT ligand stem cell factor, was detected in the MCC-1 cell line. Sequencing of KIT exon 10 in six archival MCC specimens revealed the wild-type sequence. Conclusion: The presence of the M541L KIT variation in MCC warrants further studies for its role in the pathogenesis of this tumor.*

KIT was originally described as the transforming gene in the Hardy-Zuckerman 4 feline sarcoma virus (1). The proto-oncogene *KIT*, located at chromosome 4q12, contains 21 exons and encodes a type III receptor tyrosine kinase that plays key role in various physiologic processes. Inactivating mutations of *KIT* lead to macrocytic anemia, sterility and loss of skin pigmentation (2). On the contrary, gain-of-function mutations have been implicated in the pathogenesis of various neoplasms, including gastrointestinal stromal tumors (GIST), mastocytosis, and melanoma (3-5). In addition, constitutive KIT activation by autocrine expression of its ligand, the stem cell factor (SCF), has been reported in small cell lung carcinoma (6). The importance of KIT in oncogenesis is shown by the apparent efficacy of drugs preferentially targeting mutant forms of the protein, as is the case of imatinib for the treatment of advanced, KIT-positive, malignant GIST (7).

Merkel cell carcinoma (MCC) or primary neuroendocrine carcinoma of the skin, a rare and aggressive cutaneous tumor (8, 9), has been previously reported to express the KIT receptor tyrosine kinase (10). Co-expression of KIT and its ligand have been recently described in MCC, suggesting autocrine KIT activation (12-13). Importantly, KIT expression has been associated with unfavourable prognosis in Merkel cell carcinoma, since Andea *et al.* (14) reported 0% *vs.* 57.8% survival rates of patients with tumors with high *vs.* low KIT expression, and Waltari *et al.* (15) worse survival for those with tumor KIT expression when stratified by the presence of Merkel cell virus DNA. Because mutations of *KIT* have been identified in several KIT-positive tumors, many groups attempted to address the question of whether *KIT* is also mutated in MCC. These studies failed to reveal the presence of *KIT* mutations that result in protein sequence alterations (12, 14, 16-17). However, their search was limited to exons 9, 11, 13, 17, regions that commonly harbor *KIT* mutations in cancer. Since mutations in other regions of *KIT* have not yet been investigated in MCC, we thus aimed in the present study to further screen for *KIT* mutations in MCC. As studies on archival tissues are often limited by the paucity of material and low quality of DNA for sequencing purposes, we analyzed the entire coding

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Table I. *Primer sets and conditions for PCR amplification of human KIT exons.*

Exon	Exon size (bp)	Forward primer	Reverse primer	PCR product size (bp)	Annealing Temp. (˚C)	Seq. primer	Sequencing primer
1	154	TTTGCCGCGCTCGCTGCAC	TGCTGTCCCCATTCCCGAAG	391	54	1	TTCGCCGAGTAGTCGCAC
2	270	GGCATTCAGCACATCCTGGC	AGTATCACAGTCCTGCAGTC	739	56	2	GCTTTATTTCGCCAAGG
3	282	TGGTCCAAGCTTAGTGCGTG	ATGAGAGGCAGTCATGGAAC	698	56	3	CCACTAGTCATGAAAGGC
4	137	GCTGGTACCTTCAGATATGC	CTGGCCTAAACGGTATCAAC	662	56	4	GCTGTACACATTTGAGG
5	169	GTGTTCCAATGACAGACTTG	GCTTTCATTGCAAGAGGC	502	56	5	ATCTAGGAAAGATTCTG
6	190	CTCACTTGTGACAGTGATTC	AGTGTGCATGCTCTAAACAC	556	56	6	TCCAAGATGAGGTTCTG
7	116	TCTCTCACAACAGGCGTTGG	CCACGAAGTCTCTGTGAAGG	461	56	7	GCTATCCACAGGTGATTG
8	115	AACACTTTGGAGTCCTAGAG	ACCTGCAGGCTAGAAATTGC	709	56	8	CTGAGGTTTTCCAGCAC
9	194	AGGATTCAAACCCGCATCTG	GCTCTGGTAAAAGCATATAC	608	56	9	GTATGCCACATCCCAAG
10, 11, 12, 13	107, 127, 105, 111	GTAACCAAGGTGAAGCTCTG	TAACAGAAGCCTTCTGGTCG	1341	56	10 a 10 b	TGTGGTAGAGATCCCATC GGTTTGCCATAGAGAAC
14	151	GTTACTCCACATAAGGCTGC	ATGCATGTAGTACCAAGCTC	672	56	11	TTAATGGCCATGACCAC
15	92	AAGGACACCTAGTTTCTGGG	GAGACAGCAGTTGGAACATG	1072	54	12 a	GAGTGCCCTTCTACATG
16	128	AGCAGTGCCAATGGTCAATG		639	54	12 b	AGTGATCTGCCTGCAAG
17	123	CATGGGTGACATTTCCCAAC	CTGCTGTGACCTTCAATGAC	924	54	13	CATCATTCAAGGCGTAC
18, 19, 20	111, 100, 106	GGATAATGCACTTGTGAGCC	ATCCTTCTTTGGCTCAGAAC	1184	54	14a 14b	ATTTCAGCAACAGCAGC TTGCTGGATGCCCATAC
21	2287	GGCCACAAAGTTCTTGGAAAC	GCTTGTTCCACAGAATCTAC	1178	56	15a 15b 15c	GACACTGTAAGTATGCC ACGTAGCTTCTACCATG AGCACTTGTATATACGC
		CTGGCATTATGTCCACTGTG	GGTGCGAGAGCATAGAACTC	930	54	15d	TAGCTGTCTAGAGTAGC
		TACATTTAGAGAACTGTGGCC	CAATCAAATGACTCCACTTG	596	56	15e 15f	TGTCACCCAAGAGATTG GAATTCTTCAGAACTGTC

Forward, reverse and sequencing primers are shown. The size of exons and PCR products, as well as the annealing temperatures are indicated.

region of *KIT* in the Merkel cell carcinoma cell line MCC-1 (18). Subsequently, 21 archival MCC tumor specimens were processed for DNA extraction and examined for *KIT* sequence variations found in the cell line.

Materials and Methods

Cells. MCC-1, a Merkel cell carcinoma cell line with functional KIT receptor, was used for the experiments (18-19). Cells were cultured in RPMI-1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (Biochrom KG) and antibiotics (Gibco, Paisley, UK) and processed for DNA isolation and *KIT* exon mutation analysis.

Tumor specimens. Twenty-one paraffin-embedded archival MCC tissue specimens from nineteen patients were processed for DNA extraction and *KIT* exon 10 mutation analysis. Institutional Review Board approval was obtained for the study. No patient had neuroendocrine carcinoma of the lung or of the gastrointestinal tract. The diagnosis was made in all cases by routine histological examination supplemented with immunohistochemistry for typical dot-like immunoreactivity with antibodies directed against cytokeratins and for neuroendocrine markers, such as neuron-specific enolase, synaptophysin, chromogranin and neurofilaments.

DNA extraction and polymerase chain reaction (PCR). Genomic DNA was extracted from the MCC-1 cell line using standard procedures. Briefly, cells were lysed in buffer containing 50 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% SDS and 0.5 mg/ml proteinase K. After digestion with RNAase A, DNA was isolated by phenol/chloroform/ethanol extraction. The quantity and quality of DNA were determined by spectrophotometry. After isolation, genomic DNA was used as template for amplification by PCR of exons 1-21 of *KIT* gene in the MCC-1 cell line. Specific primers for exon amplifications were designed based on the human sequence (Genbank accession number HSU63834, chromosome 4) (Table I).

Genomic DNA was extracted from formalin-fixed paraffin-embedded tissue blocks using a QIAamp DNA FFPE tissue DNA kit (Qiagen, Hilden, Germany). Five to eight 5 μm-thick sections were cut from each paraffin block. DNA of sufficient quantity and quality was obtained from 6 of the 21 initially processed biopsies. Genomic DNA was used as template for PCR amplification of exon 10, using two pairs of primers (nested PCR). The following primer sequences were used: forward: GTAACCAAGGTGAAGCTGTG and reverse: TCCCATTTGTGATCATAAGG (first PCR) and forward: CACATAGCTTTGCATCCTGC and reverse: CCTCAACAACCTTCCACTGT (second PCR). PCR amplifications were carried out in 50 μl in a PTC-200 thermal cycler (M.J. Research, Inc., Watertown, MA, USA), using a Qiagen Taq PCR Core Kit (Qiagen).

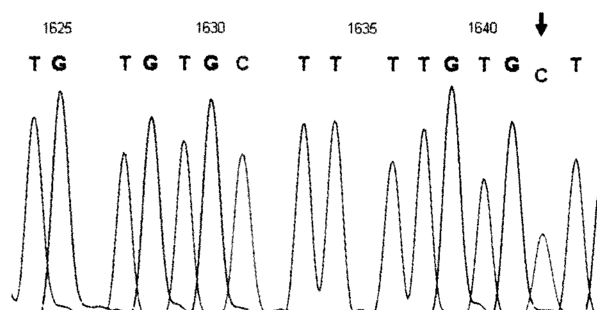

Figure 1. *Electropherogram demonstrating the A>C transition at nucleotide 1642 of the KIT coding region, resulting in the substitution of methionine for leucine at amino acid position 541.*

Table II. *KIT sequencing findings in the Merkel cell carcinoma cell line MCC-1.*

Position*	Substitution	Homo/Heterozygous	Intron/Exon
68245	T → C	Hetero	Intron 9
69384	A → C	Hetero	Exon 10
69785	A → G	Hetero	Intron 10
69821	C → T	Hetero	Intron 10
73906	T → A	Hetero	Intron 12
75078	A → G	Hetero	Intron 13

*Position in the genomic sequence (Genbank accession number HSU63834).

Direct DNA sequencing. The PCR products were separated by agarose-gel electrophoresis and isolated from gel slices using either a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) for the MCC-1 cell line, or QIAquick Gel Extraction Kit (Qiagen) for tissue samples, according to the manufacturer's instructions. Direct single-strand sequencing of PCR products for the MCC-1 cell line and double-strand for tissue samples were performed by the Sequencing Facility of the Institute of Molecular Biology and Biotechnology (Heraklion, Greece). Analysis of the sequence data was performed using the BLAST program (NCBI, U.S. National Library of Medicine, Bethesda, MD, USA). *KIT* sequence alterations were confirmed by double-strand sequencing.

Results

KIT sequence variations in the MCC-1 cell line. Genomic DNA from the MCC-1 cell line was used to determine the sequence of the entire coding region in exons 1-21 of the human *KIT* gene. All exons were successfully amplified by PCR using the primers in Table I, and PCR products had the expected sizes. Sequencing of the products detected a single base pair change in one allele of exon 10 in the transmembrane *KIT* domain. Repeated amplifications followed by direct, double-strand sequence analysis proved a single base transition from adenine to cytosine (A>C) at position #1642 (Figure 1). This transition results in an amino acid substitution of methionine to leucine at position #541 in the transmembrane domain of KIT (M541L). Several further sequence alterations were detected in *KIT* introns, outside the coding region (Table II).

Sequencing of KIT exon 10 from Merkel cell carcinoma biopsies. Due to the identification of the KIT M541L substitution in exon 10 in the MCC-1 cell line and given that previous investigations did not reveal any *KIT*-activating mutations in exons 9, 11, 13 or 17 (12, 14, 16-17), we were prompted to screen exon 10 for the A1642C sequence alteration in MCC tumor specimens. Therefore, 21 MCC tumors were processed for DNA isolation and *KIT* exon 10 sequencing. In six tumors originating from five MCC patients, DNA of sufficient quality and quantity was isolated and *KIT* exon 10 was studied by direct sequencing. All tumors were found to carry the wild-type *KIT* sequence in exon 10.

Discussion

Genetic analysis of *KIT* in the Merkel cell carcinoma cell line MCC-1 revealed the presence of the A1642C sequence variation which results in the M541L substitution of the *KIT* transmembrane domain. Several studies have implicated this *KIT* variation in the development of neoplasia. More specifically, the same base abnormality at codon 541 was detected with higher frequency in chronic myelogenous leukaemia patients (20) than in the general population (21-23). Interestingly, in one of the CML patients, the abnormality developed during blastic crisis and was not present in leukemia cells of the chronic phase nor in normal cells of the same patient. The M541L substitution was detected in all dedifferentiated locally aggressive liposarcomas but only in one third of the well-differentiated tumors (24). It was also found in 28% of biopsies in pediatric mastocytosis together with other *KIT* mutations but was absent from patients' genomic DNA (25). In another study, the M541L variant was detected in 18% of mastocytotic lesions but not in the patients' blood samples and only in 1.5% of the healthy population (26). These findings suggest that the M541L substitution may represent a true somatic event in cancer and an increasing number of cancer studies now routinely screen for this variant in their samples (25, 27-28). However, it is worth pointing out that because *KIT* exon 10 is not a mutation hot spot and has not therefore been routinely screened, the frequency of the M541L substitution may be well underestimated in cancer.

Two studies have shown that the M541L substitution alters KIT receptor activity. Inokuchi *et al.* (20) reported that lymphoid cells expressing the respective murine KIT M540L substitution had higher levels of tyrosine phosphorylation and elevated proliferation response to low levels of SCF

compared to wild-type cells. Foster *et al.* (29) reported similar findings upon expression of the human KIT M541L receptor in myeloid progenitor cells. It is important to note that the M541L substitution in the absence of ligand does not permit KIT receptor activation or cell proliferation. Nevertheless, as autocrine production of SCF is common in MCC (13), the M541L substitution may be of significance for its pathogenesis. Upon co-expression of SCF and KIT receptor, this variation may provide tumor cells with a growth advantage under conditions of low SCF production. Indeed, we demonstrated an autocrine stimulatory loop between SCF and KIT in the MCC-1 cell line which carries the M541L sequence variation (19).

Our study may not have detected the *KIT* M541L sequence variation in Merkel cell carcinoma biopsies due to the small number of tumors that provided adequate DNA for sequencing. Therefore, it might be of particular interest to further search for this sequence variation in a larger number of tumor samples, considering that the *KIT* M541L variant may be a pathogenic factor for a subset of Merkel cell tumors.

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