The oncogenic *B-raf* V599E mutation occurs more frequently in melanomas at sun-protected body sites

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Abstract. Downstream of Ras, the serine/threonine kinase, B-raf, has recently been reported to be mutated, among other carcinomas, in a substantial subset of primary melanomas with a preponderance of the oncogenic V599E transition. As the risk of melanoma is enhanced by intermittent ultraviolet (UV) exposure but is less with chronic UV exposure, we here studied *B-raf* kinase domain (exon 15) mutations in primary cutaneous melanomas with respect to anatomical locations reflecting chronic versus intermittent UV exposure. Investigating a representative number of 101 primary melanoma resection specimens for the presence of mutations within the activation segment (exon 15) of the *B-raf* kinase domain by polymerase chain reaction and single-strand conformation polymorphism gel electrophoresis, followed by DNA cloning and sequencing, we found 32 cases (32%) which harbour somatic *B-raf* exon 15 mutations. As to the B-raf protein sequence, the V599E mutation was predicted in 66% of these positive melanomas, followed in frequency by the V599K transition (16%). Only two C→T transitions, considered to be induced by UV irradiation, occurred in two melanomas located on the head. Among 23 melanomas located at body sites with chronic UV exposure, only a single tumour harboured the B-raf V599E mutation (4%), which was a significantly lower frequency in comparison to melanomas from sun-protected body sites (26%; Fisher's exact test, p=0.038; odds ratio, 7.59). Our observation parallels the epidemiological data of intermittent sunlight exposure on unacclimatised skin increasing the risk of melanoma development.

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Introduction

Cutaneous melanoma is the most lethal skin cancer and its incidence has risen rapidly in recent decades (1). A majority of melanoma cell lines have been described to harbour mutations of the oncogene encoding the serine/threonine kinase, B-raf, which participates in the RAS-RAF-mitogen-activated protein (MAP)-kinase kinase (MEK)-extracellular signal-regulated kinase (ERK)-MAP kinase pathway of signal transduction (2). Remarkably, all mutations were within the kinase domain, with GTG→GAG transversion at codon 599 leading to a valine to glutamic acid exchange at this position (V599E) accounting for 95% of *B-raf* mutations in melanoma cell lines and leading to constitutive kinase activity (2). Addressing primary cutaneous melanomas, 5/9 (2), 4/5 (3) and 12/50 (4) of the resection specimens harboured this V599E mutation. Analyzing primary tumours as well as metastases in the same 51 patients, all except two cases showed the same *B-raf* genotype in primary lesions and corresponding metastatic tissue (5).

Notably, V599E is the first mutation of an oncogene described for a relevant proportion of sporadic melanomas, which constitute over 90% of all melanoma cases and which are linked to sunlight exposure (6-9). As the risk for melanoma is enhanced by intermittent ultraviolet (UV) exposure (10) but is less with chronic UV exposure (11,12), we here studied *B-raf* kinase domain mutations in resection specimens of primary cutaneous melanomas with respect to anatomical locations reflecting chronic versus intermittent UV exposure.

Materials and methods

Isolation of genomic DNA from paraffin-embedded tissue. Total cellular DNA was extracted from paraffin-embedded melanomas of the skin. DNA was isolated from both tumour and surrounding skin tissue by microdissection. Slides with 15-μm tissue sections were incubated in xylene for 30 min and in a series of 100%, 80%, 60%, 40% ethanol and in aqua for 10 sec each at room temperature. Tissue was scratched from the slides under microscope and placed in tubes, and isolation of DNA was performed using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) according to the

manufacturer's instructions. Briefly, 25 mg tissue was deparaffinized in 1.200 µl xylene, pelleted by 5-min full-speed centrifugation, washed in ethanol, resuspended in buffer ATL and digested by proteinase K. Following incubation in buffer AL at 70˚C for 10 min, ethanol was added. DNA was absorbed at QIAamp spin columns by centrifugation at 8.000 rpm for 1 min. The flowthrough was discarded and the tube was centrifuged at 8.000 rpm for 1 min with 500 µl washing buffer AW1 and for 3 min at 14.000 rpm with washing buffer AW2. DNA was eluted with 100 µl distilled water by centrifugation at 8.000 rpm for 1 min. The quality of the isolated DNA was assessed by agarose gel electrophoresis.

Polymerase chain reaction (PCR). Prior to PCR-SSCP analysis of *B-raf* exon 15 in melanomas, a 184-base pair (bp) fragment of the human *A-myb* gene was amplified by primers A-Myb-1 and A-Myb-2 to ensure the DNA integrity of the genomic DNA samples as well as the absence of major Taq polymerase inhibitors. PCR was performed in a final volume of 25 µl containing 0.6 units proofreading FailSafe Taq DNA polymerase and 12.5 µl FailSafe PCR 2X PreMix F (Biozym, Oldendorf, Germany), containing 100 mM Tris-HCl pH 8.3, 100 mM KCl, 400 µM of each dNTP, supplemented by 0.25 µl of each primer (100 pmol/µl). PCR conditions were 95˚C for 2 min; 46 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec; followed by 72˚C for 7 min. All PCRs were performed on a GeneAmp 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). PCR products were analyzed by 1.5% agarose gel electrophoresis.

To screen for mutations in the activation segment (exon 15) of the *B-raf* kinase domain, 100 ng of each melanoma DNA sample was PCR amplified by primers B-raf-6998-for and B-raf-70221-rev in 25 µl PCRs as described above. Aliquots (5 µl) of each reaction were electrophoresed on 1.5% agarose/ethidiumbromide gels using highly resolving NuSieve 3:1 agarose (Biozym). Primer sequences according to the database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA, http://www.ncbi. nlm.nih.gov/BLAST) were as follows: A-Myb-1, 5'-CAT ggA ATg CCA ATT TAA Cg-3'; A-Myb-2, 5'-CAT CCC TAA gTT CgC TgC C-3' (gene database accession number X66087); B-raf-6998-for, 5'-ggC CAA AAA TTT AAT Cag Tgg A-3' (identical with primer sequence exon-15-rev according to ref. 21); B-raf-70221-rev, 5'-TCA TAA TgC TTg CTC TgA TAg gA-3' (identical with primer sequence exon-15-for according to ref. 21; gene database accession number NM_004333).

SSCP gel electrophoresis. Amplified DNA was mixed with an equal volume of formamide loading dye (94% formamide, 0.05% xylene cyanol and 0.05% bromphenol blue), denatured at 95˚C for 5 min, chilled on ice for 1 min and loaded onto Gene Excel 12.5/24 polyacrylamide gels (Pharmacia Biotech, Freiburg, Germany). Following non-denaturing electrophoresis at 600 V, 25 mA, 15 W, 6˚C for 80 min, DNA fragments were stained by silver using a DNA silver staining kit according to the manufacturer's protocol (Pharmacia Biotech). DNA fragments that reproducibly showed mobility shifts according to independent two-repeated SSCP analyses were excised from the acrylamide gel and subjected to semi-nested PCRs performed with primers B-raf-70023-for and B-raf-70221-rev using the conditions described above. The sequence of primer B-raf-70023-for was 5'-ATA gCC TCA ATT CTT ACC ATC C-3'.

Cloning of PCR amplicons and sequencing of plasmid inserts. Nested PCR products were then purified using the QIAquick PCR purification kit (Qiagen) and cloned into pCR^{R}2.1-TOPO vector (Invitrogen, NV Leek, The Netherlands) according to the manufacturer's instructions. Briefly, 10 ng of the secondary PCR product were ligated into 10 ng vector and the ligation mixture was introduced into competent TOP10 bacteria by heat shock. The library was plated onto LB plates containing 50 µg/ml ampicillin. Single bacterial transformants that appeared positive in blue/white screening were picked randomly and grown in 2 ml LB medium containing 50 µg/ml ampicillin at 37˚C overnight. Following alkaline lysis of bacterial cultures, neutralized lysates were loaded onto silica-gel membranes (QIAprep spin miniprep kit, Qiagen) and plasmid DNAs were eluted in low-salt buffer.

Plasmid inserts were sequenced at a concentration of 50 ng/µl with a GeneAmp PCR system 9600 using ABI PRISM dGTP BigDye terminator ready reaction kits and AmpliTaq DNA polymerase FS according to the manufacturer's protocol (Perkin-Elmer, Seqlab, Göttingen, Germany). For sequencing *B-raf* exon 15 fragments from both sides, M13 forward and M13 reverse primers were used. PCRs consisted of 25 cycles including a denaturation step at 96˚C for 10 sec, a primer annealing step at 50˚C for 5 sec and a chain elongation step at 60˚C for 60 sec. Cycle sequencing products were then ethanol precipitated, run on a 4% polyacrylamide 7 M urea gel and analyzed using an ABI PRISM 377 genetic analyzer (Perkin-Elmer). Primer sequences were as follows: M13 forward primer, 5'-CAA AAg ggT CAg TgC Tg-3'; M13 reverse primer, 5'-gTC CTT TgT CgA TAC Tg-3'. The resulting sequences were aligned to the known *B-raf* sequence in the NCBI database (accession no. NM_004333). The numbering began with the start codon ATG, corresponding to nucleotide positions 1-3. B-raf protein sequences predicted by the cDNA sequences were compared with B-raf wild type protein sequence (NCBI accession no. NP_004324) using the BLASTX software at the NCBI.

Statistical analyses. Melanomas localized on the head, face or dorsal hands and therefore chronically exposed to UV radiation were compared for the frequency of *B-raf* V599E mutation with melanomas at all other anatomical sites using the χ^2 test. With more than 20% of the expected frequencies smaller than 5, the χ^2 test was not regarded to be valid for testing the data and we conducted Fisher's two-sided exact test for 2x2-tables or an exact test for higher-order rxc-tables. To confirm that UV exposure and not histological type of melanoma is responsible for the lower V599E frequency, the χ^2 test and exact tests for rxc-tables were also applied to compare melanomas from UV-exposed skin with melanomas from sun-protected anatomical sites for histological characteristics (histological type, Clark's level) and to exclude an association between histological melanoma type and the probability of B-raf exon 15 mutation. The exact Wilcoxon's rank sum test was applied to exclude the hypothesis of *B-raf* exon 15 mutations being associated with tumour thickness

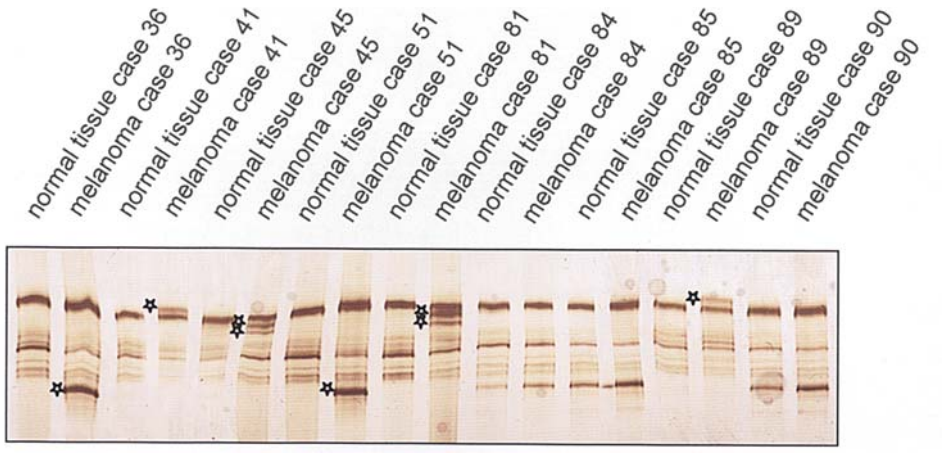

Figure 1. PCR-SSCP analysis of *B-raf* exon 15 in resection specimens from primary cutaneous melanomas. Cases 36, 41, 45, 51, 81 and 89 exhibit different DNA fragment mobilities when compared to the respective normal tissue from patients.

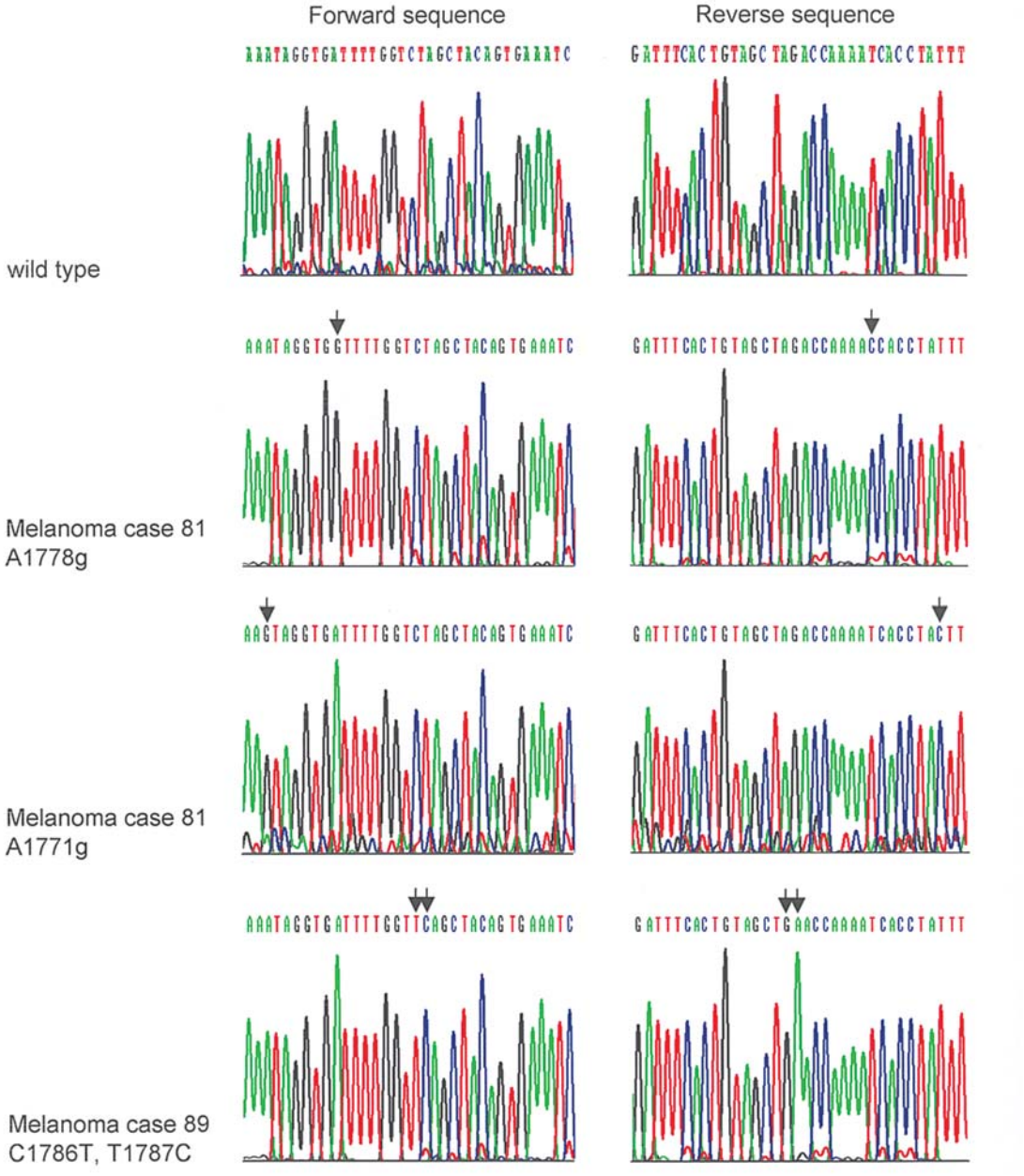

Figure 2. Part of the DNA sequence of *B-raf* exon 15, harbouring missense mutations in PCR amplicons from representative melanoma cases 81 and 89 (upper panel, wild-type DNA sequence; left, mutations in the sense strand; right, mutations in the antisense strand).

according to Breslow, which is the most important parameter for melanoma prognosis (13). As *B-raf* mutations in melanomas have recently been associated with lower age at diagnosis when compared with cases without mutations (14), we used logistic regression to test this hypothesis.

We report two-tailed statistics throughout. An association was considered as statistically significant if the p-value of its corresponding test statistic was ≤ 5%. For rxc-tables with >20% of the expected frequencies <5, we calculated the exact test with the software package StatXact-5, release 5.0.3 (Cytel Software Corporation, Cambridge, MA, USA). All other statistical calculations were performed using the statistical software SAS, release 8.02.

Results

B-raf exon 15 mutations in primary melanomas focus on T1796A. Testing resection specimens of primary melanomas of the skin for presence of amplifyable DNA by PCR amplification of a 184-bp human *A-myb* gene fragment, 101 specimens (42 superficial spreading melanomas, 19 nodular melanomas, 9 acrolentiginous melanomas, 5 lentigo maligna melanomas, 26 melanomas not further classified) were positive for both cancerous and matched non-cancerous tissues and were therefore included in further analysis. Positive results in A-myb PCR were in accordance with agarose gel electrophoresis showing the integrity of the high molecular weight DNA. Analyzing these 101 melanoma resection specimens by PCR and SSCP gel electrophoresis for mutations in exon 15 of the *B-raf* gene, 32 (32%) cases (16 superficial spreading melanomas, 7 nodular melanomas, 2 acrolentiginous melanomas, 1 lentigo maligna melanoma, 6 melanomas not further classified) exhibited SSCP patterns distinctly different to those obtained from corresponding adjacent normal tissue (Fig. 1).

Sequencing of cloned PCR-SSCP amplicons resulted in a total of 64 DNA point mutations of *B-raf* exon 15 (Table I). The activating mutation, T1796A (2), was present in 26 (81%) of the 32 melanoma resection specimens harbouring *B-raf* exon 15 mutations (Fig. 2), followed in frequency by the G1795A mutation in 6 cases (19%) and T1746C, A1773g, A1755g and T1781C mutations in 2 cases each (6%). Altogether, 29 different *B-raf* exon 15 mutations (A1743g, T1746C, T1749C, A1755g, A1771g, A1773g, A1778g, A1788g, T1781C, A1786g, g1787A, g1789A, g1795A, T1796A, A1800g, T1807g, A1810g, A1810T, g1811T, g1811A, g1814A, A1820g, T1826C, T1837C, T1839C, g1840A, T1843C, C1845T, T1850C) were detected in the 101 investigated cutaneous melanoma resection specimens. In all melanomas, the mutations in neoplastic tissue were shown to be somatic by cloning and sequencing of SSCP gel bands from corresponding normal tissue.

It is remarkable that CC→TT or C→T transitions, which occur in the *p53* gene in non-melanoma skin cancers following exposure to UV light (15-18) occurred in 2/23 melanomas located at chronically UV-exposed body sites (case 89, C1786T; case 98, C1845T) but in none of 78 melanomas at sun-protected body sites.

B-raf protein mutations in primary melanomas focus on V599E. The B-raf protein sequences predicted by the nucleotide sequences that we found were compared with the known B-raf protein sequence (NCBI accession no. NP_004324) using BLASTX software. All melanomas harbouring *B-raf* point mutations, except case 19, were predicted to exhibit alterations of the protein sequence (Table I). Within the kinase domain, the single substitution V599E occurred in 21 of the 31 positive melanomas (66%), followed in frequency by V599K in 5/31 (16%) cases. Valine at protein sequence position 599 was replaced in a total of 27/31 (87%) melanoma resection specimens. No non-sense mutation of *B-raf* exon 15 DNA, causing a predicted protein truncation, occurred in our analysis.

B-*raf V599E mutations more frequently in melanomas from sun-protected body sites*. Among the 101 investigated primary melanomas, 23 tumours were located on the head (10 cases), face (11) or dorsal hands (2). These anatomical locations were regarded as body sites with chronic UV exposure, which was confirmed by the finding of solar elastosis on histological evaluation. Among these 23 cases, only a single tumour harboured the B-raf V599E mutation (4%), whereas 20/78 (26%) primary melanomas from sun-protected anatomical locations were positive, which was a statistically significant difference upon Fisher's exact test (p=0.038). The odds ratio was estimated as 7.59; the chance of B-raf V599E positivity therefore is almost 8-fold higher for primary melanomas at sun-protected sites when compared with melanomas at anatomical locations with chronic UV exposure.

As the 23/101 (23%) melanomas located at the head, face or dorsal hands included 4/5 (80%) of the lentigo maligna melanomas but only 6/36 (17%) of the superficial spreading melanomas and none of the 9 acrolentiginous melanomas (0%), the distribution of histological melanoma types between the head, face, dorsal hands and all other anatomical sites differed significantly upon the exact test (p=0.0032). Thus the histological type of melanomas depends on the anatomical site. We therefore tested a possible association between the type of melanoma and the frequency of V599E mutations, which could be excluded by the χ^2 test (p=0.39) and the exact test (p=0.40).

The frequency of B-raf V599E mutations was not statistically associated with tumour thickness according to Breslow (two-sided Wilcoxon's test: p=0.65) or with Clark's invasion level (χ^2 test: p=0.31; exact test: p=0.36).

Besides the B-raf V599E mutation, the 101 primary melanomas that we investigated harboured V599K as the second most frequent mutation, which occurred in 1/23 primary melanomas from the head or dorsal hands (4%) and in 4/78 melanomas from other anatomical sites (5%), which was no significant difference upon χ^2 test (p=0.88) or Fisher's exact test (p=1.00).

In contrast to a previous report (14), we could not confirm *B-raf* V599E mutations to be associated with lower age at diagnosis when compared with wild-type cases. A logistic regression of the probability for a B-raf V599E mutation yielded a low explanatory value of the variable age (p=0.569).

Discussion

Investigating primary melanomas with respect to the anatomical location, we here describe the activating V599E mutation of

Table I. *B-raf* exon 15 mutations in resection specimens from primary cutaneous melanomas and predicted protein sequence alterations.[a]

Case	Type	Breslow	Clark	Location	DNA mutations	Predicted protein changes
4	ALM	1.7	IV	Plantar	T1796A, A1820g	V599E, H607R
11	ALM	1.1	IV	Heel	T1796A	V599E
13	NM	2.4	IV	Vulva	T1796A	V599E
14	SSM	1.9	IV	Gluteal	T1796A	V599E
17	MM	8	V	Foot	T1796A	V599E
					g1789A	A597T
19	NM	13	V	Toe	A1755g	WT
22	MM	2	IV	Plantar	T1796A	V599E
					T1826C	F609S
24	SSM	0.7	IV	Foot	T1796A	V599E
28	SSM	1.5	V	Abdomen	A1788g, T1807g	W603G
					A1773g, T1796A	I591M, V599E
36	NM	5.9	IV	Back	T1781C, T1796A	F594S, V599E
41	MM	1.3	IV	Lower leg	A1743g, T1796A	V599E
42	SSM	2.4	IV	Back	g1795A, T1796A, g1814A, T1837C	V599K, G605E, S613P
43	SSM	1	IV	Back	T1796A	V599E
45	SSM	3.4	IV	Lower leg	T1746C	WT
					T1796A	V599E
46	SSM	4.5	III	Back	g1795A, T1796A	V599K
47	SSM	1.8	IV	Lower leg	T1796A, A1810T, g1811T	V599E, S604F
50	NM	2.3	IV	Abdomen	g1795A, T1796A, T1850C	V599K, L617S
					g1795A, T1796A, T1843C	V599K, S615P
51	NM	2.3	V	Abdomen	T1746C, g1795A, T1796A	V599K
55	MM	1.8	IV	Shoulder	T1796A	V599E
58	SSM	1.4	IV	Shoulder	T1796A	V599E
59	SSM	1.3	IV	Upper arm	T1796A	V599E
62	SSM	2.2	IV	Back	T1796A	V599E
					g1811A	S604N
64	SSM	4.4	IV	Shoulder	T1796A	V599E
66	SSM	2.2	IV	Forearm	A1810g	S604G
71	SSM	0.8	IV	Abdomen	T1796A	V599E
					A1755g, T1796A	V599E
77	SSM	0.95	IV	Abdomen	T1796A	V599E
79	NM	3.6	V	Head	T1749C, g1795A, T1796g	V599R
80	MM	2	IV	Face	T1796A	V599E
81	LMM	2.8	IV	Face	A1778g	D593G
					A1771g	I591V
83	SSM	2.4	IV	Head	g1795A, T1796A	V599K
					g1840A	G614R
89	MM	1.8	IV	Head	C1786T, T1787C	L596S
98	NM	2.8	IV	Head	T1839C	WT
					A1800g	WT
					A1773g, T1781C, C1845T	F594S

[a]Distinct mutations in two different abnormal bands in SSCP gels are specified by two lines for one melanoma. Abbreviations: A, adenine; T1796A, change from thymine to adenine at nucleotide 1796 (according to NCBI accession number NM_004333, the start codon ATG corresponding to nucleotide positions 1-3); ALM, acrolentiginous melanoma; Breslow, tumour thickness according to Breslow; Clark, invasion level according to Clark classification; C, cytosine; E, glutamine; F, phenylalanine; G, glycine; g, guanine; H, histidine; I, isoleucine; K, lysine; L, leucine; LMM, lentigo maligna melanoma; M, methionine; MM, melanoma not further classified; NM, nodular melanoma; P, proline; pred, predicted; R, arginine; S, serine; SSM, superficial spreading melanoma; T, thymine (DNA), threonine (protein); V, valine; V599E, substitution of valine to glutamic acid at codon 599 (according to NCBI accession number NP_004324.1); W, tryptophane; WT, wild-type of protein sequence.

the *B-raf* oncogene to occur significantly more frequently in sporadic melanomas arising at body sites which are usually sun protected but exposed to intermittent UV exposure during recreational sun bathing. In comparison, the chance of B-raf V599E positivity is approximately 8-fold lower for primary melanomas at anatomical locations with continuous UV exposure.

Our observation is in line with the epidemiological data on melanoma risk where intermittent sun exposure and repeated sunburns in childhood support the development of melanoma (10,19) whereas chronic sun exposure decreases the relative risk, at least in temperate climates like Canada and Denmark (11,12,20,21). Melanomas are therefore not simply caused by accumulating UV dose (19), lentiginous melanoma being an exception as this histological subtype is associated with chronic sun exposure and more frequently occurs in the elderly (22).

The centrality of *B-raf* in melanoma biology has been underlined by the persistence of such mutation through metastasis (5). As the B-raf^{V599E} mutant possesses 10-fold greater basal kinase activity and induces focus formation in NIH3T3 cells 138 times more efficiently than does wild-type B-raf (2), relevance of this alteration in the development of melanomas is very likely. For the growth of cancer cell lines with the V599E mutation, RAS function was not required (2). In addition to the V599E transition (GTG→GAG transversion at codon 599), the V599K mutation (tandem GTG→AAG mutation at codon 599) also leads to constitutive kinase activity and transforms NIH3T3 cells (23).

The oncogenic mutations in the kinase domain of *B-raf* result in activation of the Ras-Raf-MEK-ERK-MAP kinase cascade (24). Mutations of the *N-Ras* (25) and *H-ras* (26) genes have been reported more frequently in melanomas on sun-exposed sites; still mutations of *N-ras* occurr in <15% of uncultured melanoma tissue specimens (27-29). Downstream of Ras and upstream of MEK lies B-raf, which belongs to the Raf family of serine/threonine kinases regulated by binding RAS which is composed of the ubiquitously expressed Raf-1 and by A-Raf and B-raf (24). RAF proteins phosphorylate MEK1/2, which in turn phosphorylates ERK1/2.

The high frequency of *B-raf* mutations in melanomas may be related to a principal melanocyte-specific signalling pathway controlling proliferation and differentiation: α-melanocyte stimulating-hormone (α-MSH) and proopiomelanocortin-derived peptides, secreted by keratinocytes, bind to the melanocortin receptor I on melanocytes, leading to increased proliferation and melanogenesis in response to UVB radiation (30). This signalling cascade via stimulation through G-protein coupled receptors (GPCRs) and upregulation of cyclic AMP (cAMP) does not require RAS but also activates B-raf and subsequently ERK (31). When activated, extracellular signal-regulated kinases (ERKs) translocate to the nucleus where they regulate gene expression, leading to cell proliferation. The activation of ERKs by cAMP has been reported in a limited number of cell systems, including B16 melanoma (32).

Endothelin-1 (Et-1), a strong melanocyte mitogen (33,34), is another candidate for signalling through B-raf. Et-1, produced by keratinocytes and accentuated by exposure of keratinocytes to UVB radiation, can activate the MAP kinase pathway, although the role of B-raf in transducing this signal has not been demonstrated in melanocytes (35). Besides α-MSH and Et-1, the RAS-RAF-MEK-MAP kinase cascade is activated by further growth factors, such as basic fibroblast growth factor (36) and stem cell factor (37), leading to increased proliferation of cultured human melanocytes.

Absorption of UVB by DNA causes two types of DNA lesions, the 6-4 photoproducts and pyrimidine or cyclobutane dimers, both types of lesions can lead to genetic mutations such as C→T and CC→TT transitions as observed in non-melanoma skin cancer (15-19). It is therefore reasonable that the only two C→T transitions that we found occurred in two melanomas located on the head (cases 89 and 98). Still, the most common sequence alterations in the B-raf gene in uncultured primary melanoma samples, T1796A and g1795A, are distinct from these typical UV-associated mutations.

Altogether, we here describe the activating V599E mutation of the *B-raf* oncogene to occur significantly more frequently in sporadic melanomas arising at body sites which are usually sun protected but exposed to intermittent UV exposure during recreational sun bathing. In comparison, the chance of *B-raf* V599E positivity is approximately 8-fold lower for primary melanomas at anatomical locations with continuous UV exposure. Our observation underscores the importance of such mutations in melanoma biology and merits further investigation.

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