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Absence of BRAF Gene Mutations Differentiates Spitz Nevi from Malignant Melanoma

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Abstract. *Background: Distinction of Spitz nevus from malignant melanoma is sometimes difficult on the basis of conventional histology. A high rate of BRAF gene mutations in malignant melanomas (66%) and nevi (82%) has recently been reported. Materials and Methods: We screened a series of 20 Spitz nevi for BRAF mutations in exons 11 and 15 by denaturing gradient gel electrophoresis (DGGE). Results: BRAF mutations could not be identified in Spitz nevi. Conclusion: Our results show that mutations within the BRAF gene are useful markers for the differential diagnosis between Spitz nevus and malignant melanoma.*

Spitz nevus is a benign melanocytic tumor. Sometimes unequivocal distinction between Spitz nevus and melanoma is virtually impossible as already stated by Allen and Spitz in 1953 (1) and others (2, 3). For this reason numerous studies have attempted to find markers which are able to distinguish between these two tumor types (4, 5). At present, divergent expression of S100A6 proteins (6) and VEGF (7) have been reported in Spitz nevus and malignant melanoma.

Moreover, activating mutations of the serine/threonine kinase BRAF have recently been reported to occur in a wide range of human cancers, with the highest rate (66%) found in malignant melanoma (8-10). All mutations have been located within or near the kinase domain in exons 11 or 15.

The goal of this study was to investigate whether *BRAF* mutations occur in Spitz nevus and whether they could be used as a marker for differentiating Spitz nevus from melanoma.

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Materials and Methods

We screened a series of 20 Spitz nevi for *BRAF* mutations in exons 11 and 15 by denaturing gradient gel electrophoresis (DGGE). Considering the fact that all hitherto known oncogenic *BRAF* mutations found in cancer cell lines occured in exons 11 and 15 (8), we restricted our mutations analysis to these exons.

Tissue specimens. Twenty formaldehyde-fixed and paraffin-embedded samples containing Spitz nevi were investigated. Haematoxylin and eosin-stained sections of all samples were reviewed by one pathologist (D.M). The clinical data are reported in Table I.

Ten-μm-thick sections were mounted on glass slides. Areas of interest were microdissected manually under a light microscope. Twenty sections per nevus were used and regions containing at least 80% nevus cells were selected. The dissected areas were collected in Eppendorf tubes and deparaffinized by washing with xylene and ethanol.

Genomic DNA was extracted as described previously (11).

Controls. Five metastases of malignant melanomas were used as positive controls. DNA of blood cells of a healthy individual was included as a negative control.

DGGE-based mutation analysis. PCR using genomic DNA as template was carried out in a 50 μl mixture of 1x PCR buffer (Perkin Elmer Europe, Rotkreuz, Switzerland) containing 400 ng of template DNA, 200 μMol dNTP (Roche Diagnostics, Rotkreuz, Switzerland), 1 μM of each intron-based primer (Table II) and 1 μl of Taq Polymerase (Ampli Taq Gold, Perkin Elmer Europe). A touch-down procedure was utilized consisting of 5 sec at 95˚C, annealing for 60 sec at temperatures decreasing from 60 to 55˚C during the first 11 cycles (with 0.5˚C decremental steps in cycles 2 to 11), and ending with an extension step at 72˚C for 60 sec. Ten cycles with an annealing temperature of 55˚C and 15 cycles with an annealing temperature of 45˚C were followed by extension times of 90 sec. After a step of final extension for 10 min at 72˚C, heteroduplex formation was induced after 10-min denaturation at 98˚C by incubations at 55˚C for 30 min and 37˚C for 30 min. For DGGE, 10 μl of the PCR product were loaded with 3 μl of Ficoll based loading buffer onto 10% polyacrylamide gels containing an 0%-50% urea-formamide gradient in 0.5 X TAE. The amplicons were electrophoresed at 60˚C and 100V for 16h. The fragments

Table I. *Clinical and genetic data of Spitz nevi.*

No.	Age/Sex	Location	Exon 11 mutation	Exon 15 mutation
1	8/m	Left forearm	-	-
2	22/m	Forehead	-	-
3	5/f	Right forearm	-	-
4	10/m	Back	-	-
5	23/f	Buttock	-	-
6	3/f	Not known	-	-
7	9/m	Elbow	-	-
8	69/m	Left arm	-	-
9	5/f	Right thigh	-	-
10	12/m	Face	-	-
11	3/f	Face	-	-
12	2/m	Leg	-	-
13	10/f	Not known	-	-
14	39/f	Arm	-	-
15	35/f	Abdomen	-	-
16	28/f	Right thigh	-	-
17	17/f	Left thigh	-	-
18	15/f	Right forearm	-	-
19	11/f	Right elbow	-	-
20	34/f	Right helix	-	-

were visualized using silver staining as described (12). Samples exhibiting additional bands were sequenced on an automated ABI 377 cycle sequencer.

Results

Clinical findings. The clinical findings in 20 patients (2-69 years old) with Spitz nevi are summarized in Table I.

Histopathology. We analysed only Spitz nevi with a typical histopathology (Figure 1): symmetric compound nevus, consisting of spindle and epitheloid cells, with sharp lateral demarcation, pseudoepitheliomatous hyperplastic epidermis and signs of maturation. There was no relevant mitotic activity or pagetoid infiltration of the epidermis.

Mutation analysis. Both exons could be successfully amplified in all examined tumors. The DDGE banding pattern of the normal control DNA revealed a single dominant band. *BRAF* exon 15 DGGE analysis of 5 melanoma metastases exhibited three tumors (60%) displaying two distinct additional bands representing heteroduplexes (Figure 2, arrowhead). Sequencing of the three melanomas revealed a nucleic acid exchange T>A in codon 599 (V599E). Additional bands representing exon 15 mutations were consistently lacking in all examined Spitz nevi (Figure 2). The analysis of exon 11 showed an identical single band pattern in Spitz nevi as well as in all controls.

Table II. *DGGE primers.*

BRAF 11neuFgc	5'-*TTTTCTGTTTGGCTTGACTTGA-3'
BRAF 11 Rgc	5'-gcgcgCGAACAGTGAATATTTCCTTTGAT-3'
BRAF 15 Fgc	5'-cgcgcTCATAATGCTTGCTCTGATAGGA-3'
BRAF 15 Rgc	5'-*GGCCAAAAATTTAATCAGTGGA-3'

*: cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaa

Discussion

In the present work, we were able to show that: 1) no BRAF mutations occurred in exons 11 and 15 in this series of Spitz nevi, and 2) Spitz nevi differ from malignant melanomas, which were shown in this work and previously to carry *BRAF* mutations in up to 66% (8, 10, 13).

In addition, *BRAF* mutations were previously shown to occur in as many as 82% of other nevi, which differ from Spitz nevi in this respect (13). It was also demonstrated that a large proportion of the above-mentioned cancer cell lines (80%) carried the mutation V599E leading to a ten-fold increase of BRAF activity (8).

At present, knowledge concerning the molecular genetics of Spitz nevi is limited. The majority of Spitz nevi show a normal chromosomal complement at the level of CGH resolution. Only a subset of Spitz nevi show increased copy numbers of chromosome 11p (20% and 11.8% resp.; (14, 15)), and sequence analysis of *HRAS* revealed frequent (67%) oncogenic mutations in this subset (15). It would be important to know whether these oncogenic mutations correlate with a progression of Spitz nevus to malignant melanoma. However, the fact that a *HRAS* mutation has been found in only one out of 150 melanomas (16, 17) indicates that the presence of *HRAS* mutation in a subset of Spitz nevi does not cause an increased risk of progression to melanoma.

It is of interest that *HRAS* as well as *BRAF* belong to the RAS/RAF/MEK/ERK pathway and that *BRAF* encodes a RAS-regulated kinase, stimulating cell growth and malignant transformation by activation of the mitogen-activated protein kinase (MAPK) pathway (18).

Our results indicate, however, that activating *BRAF* mutations do not play an important role in the pathogenesis of Spitz nevi. The absence of *BRAF* mutations is in agreement with the recent results on a large series of Spitz nevi (19).

A recent publication suggested *BRAF* mutations to be neither important in melanoma initiation, nor in early melanomas with radial growth phase. In fact, *BRAF* mutations have only be found in 10% of these lesions (20). By contrast, *BRAF* mutations appear to be instrumental in

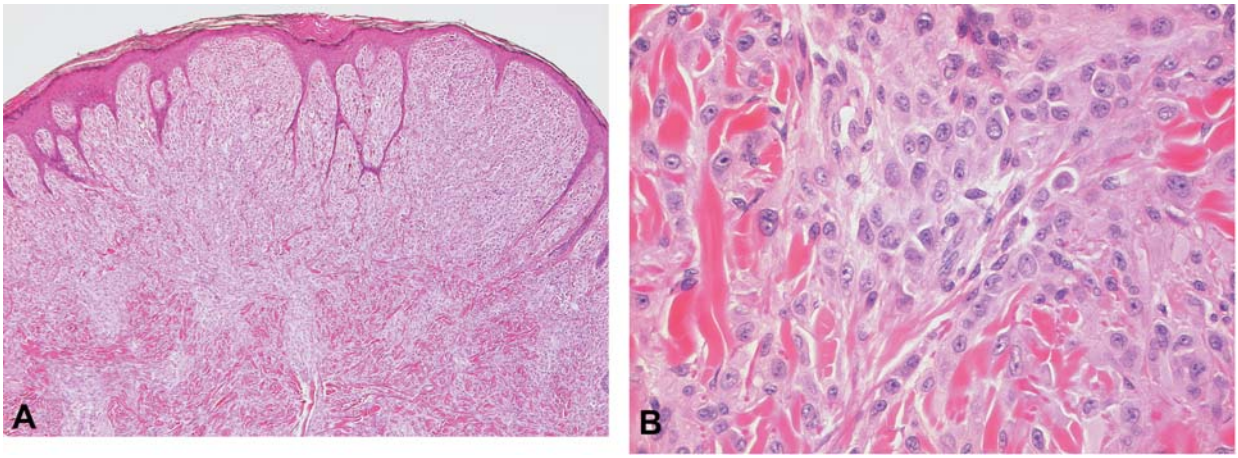

Figure 1. *Histology of Spitz nevus. A. Symmetric compound nevus with sharp lateral demarcation. Hyperplastic epidermis without pagetoid infiltration. Hematoxylin-eosin x 8. B. Epitheloid nevus cells without mitotic figures. Hematoxylin-eosin x 250.*

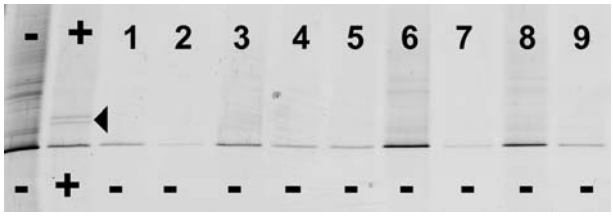

Figure 2. *BRAF exon 15 DGGE analysis with additional band in melanoma metastasis (arrow). No additional band in Spitz nevi and normal control.*

melanoma progression because the reported rate of mutations in melanomas was found to be 63% during the vertical growth phase, and in melanoma metastases *BRAF* mutations were found in 62 to 66% (8, 20). The results in the control experiments of the present study are consistent with these findings (mutations detected in exon 15 in 3 out of 5 lymph node metastases of melanomas (60%)).

We conclude that: 1) *BRAF* mutations rarely, if ever, occur in Spitz nevi, 2) the role of *BRAF* mutations in the pathogenesis of melanoma remains unknown, and 3) the exclusion of *BRAF* mutations is helpful in differentiating Spitz nevi from malignant melanoma.

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