Assessment of V600E Mutation of *BRAF* Gene and Rate of Cell Proliferation Using Fine-needle Aspirates from Metastatic Melanomas

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Abstract. *Background: metastatic melanomas are incurable by systemic treatment and it is therefore of the highest concern to develop new therapeutic regimens. RAF kinases play a key role in the RAS-RAF-MAPK signalling pathway which mediates cellular response to growth signals. An inhibitor of the RAS-RAF-MAPK cascade, sorafenib, has shown promising therapeutic results in treatment of several types of metastatic tumours. It can be hypothesized that metastatic melanomas with activating BRAF mutation may respond to RAF kinase-blocking therapy. The objective of the study was to analyze if the activating BRAF V600E mutation is present in metastatic melanomas. Materials and Methods: Fine-needle aspirates from 44 metastatic melanomas were studied. The V600E mutation in exon 15 of the BRAF gene was selected for genotyping. A Taq-Man MGB biallelic discrimination system was used. Immunocytochemical assessment of the Ki-67 antigen was used to analyze the growth fraction of cells. Results: Nearly 39% of metastatic melanomas had BRAF V600E mutation. Tumours with BRAF V600E mutation had a tendency to have a more aggressive clinical course. The growth fraction showed correlation with tumour progression. This study indicates that the V600E mutation is present in metastatic melanomas and occurs more often in sites without chronic sun exposure. Mutated tumours may have a more aggressive clinical course since such melanomas metastasize at an earlier stage. Determination of the BRAF mutation and the growth fraction of melanomas may add a prognostic value. Conclusion: A fraction of melanoma cases possess an activating BRAF mutation and may benefit from RAF-kinase inhibitor treatment. Future studies are needed to confirm this hypothesis.*

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Key Words: Fine-needle aspiration, BRAF, melanoma, proliferation.

Malignant melanomas make up a heterogeneous group of tumours characterized by particular genetic aberrations depending on their anatomic localization and UV exposure (1). The *BRAF* gene encodes a serine–threonine-specific protein kinase that acts as an intermediary in the RAS-RAF-mitogen-activated protein kinase (MAPK) signalling pathway. Activation of the MAPK signalling pathway is found in the majority of melanomas, with either somatic mutations of *BRAF* or, more rarely, *NRAS*. RAF kinases are signal-integrating enzymes that have the ability to switch tyrosine kinase signalling to serine/threonine phosphorylation and connect growth factor receptors with transcription factors. This pathway involves a cascade of protein kinases that is essential for cellular proliferation and differentiation (2). The most common BRAF mutation, V600E, mimics the phosphorylation, resulting in Ras-independent activation. In a previous study it was shown that blocking of mutant V600E B-Raf inhibited melanoma cell extravasation *in vitro* and subsequent lung metastasis development in vivo (3). Therefore, the development of RAF-kinase inhibitors for targeted therapy is of interest and might have implication in the treatment of metastatic melanomas which are today considered incurable by available methods (4-6). Agents that specifically target BRAF, such as sorafenib are being actively investigated (7, 8). The aim of the present study was to evaluate the frequency of V600E mutation of *BRAF* in cells aspirated from metastatic melanomas by fine-needle aspiration and correlate it to clinical and histomorphological properties of the primary tumours, as well as the rate of cell proliferation.

Table I. *Distribution of mutated and non-mutated cases of metastatic melanoma within groups of different cytological cell types.*

Cell type	Number of cases	% of cases
With BRAF mutation		
Epithelial cell	6	35.3
Mixed cellularity	2	11.7
Plasma cell	3	17.6
Pleomorphic cell	4	23.5
Spindle cell	2	11.7
Without BRAF mutation		
Epithelial cell	14	51.8
Mixed cellularity	5	18.5
Spindle cell	3	11.1
Plasma cell	2	7.4
Pleomorphic cell	2	7.4
Spindle cell with desmoplastic reaction	1	.7

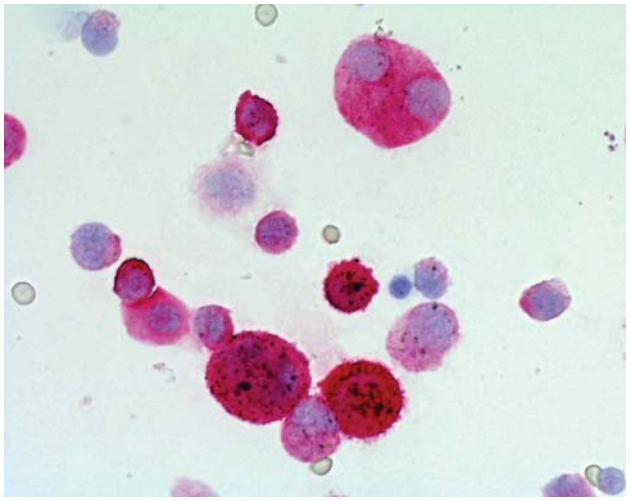

Figure 1. *HMB-45 immunostaining of metastatic melanoma (fine needle aspirate, cytospin preparation, alkaline phosphatase method, ×400 magnification).*

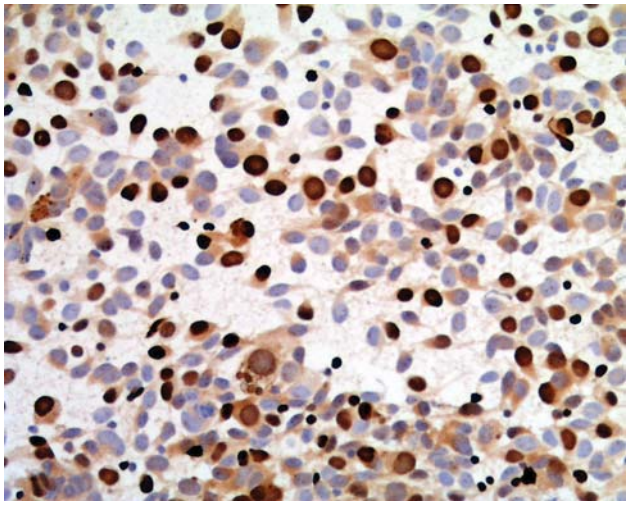

Figure 2. *MIB-1 immunostaining of metastatic melanoma (fine-needle aspirate, smear preparation, immunoperoxidase method, ×200 magnification).*

Materials and Methods

Forty-four cases of metastatic melanoma were included in the present study. The patients (25 males and 19 females) were between 28 and 90 years of age (median=68.5, mean=66.2 years). The fine-needle aspiration procedure was performed as originally described by Zajicek (9). All patients had been surgically treated for histopathologically verified cutaneous melanoma and were referred to the Division of the Cinical Cytololgy at Karolinska Hospital between 2006 and 2007 for fine-needle aspiration of lesions suspicious for recurrent disease. All cases were stratified into two groups depending on whether the primary tumours were localized on skin with chronic sun exposure/damage (wrists, head and neck) or without it (covered body parts). The metastases were localized as follows: lymph nodes, 28 cases, skin, 11 cases, skeletal muscle, 4 cases and liver, 1 case. One part of the aspirate was used to prepare smears which were subsequently stained with May-Grunwald-Giemsa method and used for cytological diagnosis. Cytologically, the metastases were descriptively classified as predominantly (more than 90% of cells) epithelial, plasma (lymphocyte-like, small cell), spindle and pleomorphic cell type, as well as mixed type (10, 11). From each aspirate, one air-dried and formalin-fixed smear was used for immunostaining with MIB-1 monoclonal antibody to the Ki-67 antigen using a peroxidase-avidin-biotin complex technique as previously described (12). Tumour cells which showed nuclear staining were scored as positive. The second part of the aspirate was suspended in phosphate-buffered saline (pH 7.4) and used for the preparation of the cytospin slides, which were used in diagnostic work-up for staining with antibodies to S-100, vimentin and HMB-45 antigens by a three-step alkaline phosphatase immunostaining procedure as described elsewhere (13). The remaining part of the cell suspension which was not used in the primary immunological work-up was frozen as a cell pellet at –20˚C and used for subsequent genotyping of *BRAF* V600 E mutation. Extraction of the genomic DNA was performed using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The concentration of DNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). A 5' nuclease assay for allelic discrimination method was used for genotyping of the *BRAF* mutation. The V600E mutation in exon 15 of the *BRAF* gene was selected for genotyping. A Taq-Man MGB biallelic discrimination system was used as previously described (14). Probes and oligonucleotides were synthesized in 40 concentrations by Applied Biosystems (Foster City, CA, USA) using the Assay-by-Design ordering system. PCRs were performed on ABI prism Sequence Detection System 7000 (Applied Biosystems). The 25 μl PCR contained 1× of the TaqMan Universal PCR Master Mix, No AmpErase UNG, 1× of the assay probe and primer mix (forward primer CATGAAGACCTCACAGTAAAAATAGGTGAT; reverse primer GGATCCAGACAACTGTTCAAACTGA; VIC

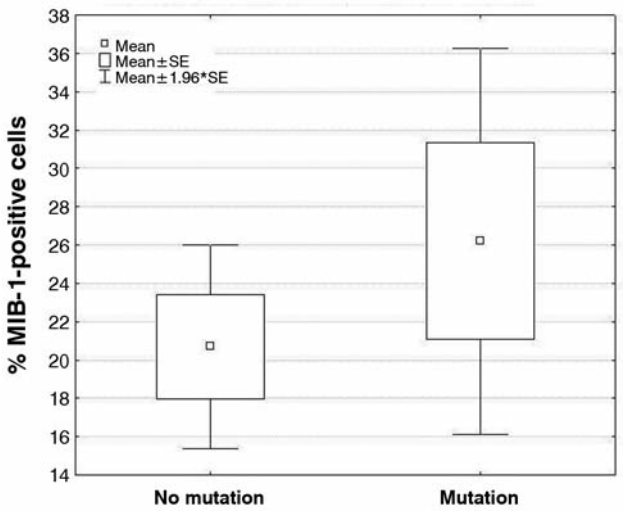

Figure 3. *Growth fraction as assessed by MIB-1 in metastatic melanoma with and without BRAF V600E mutation.*

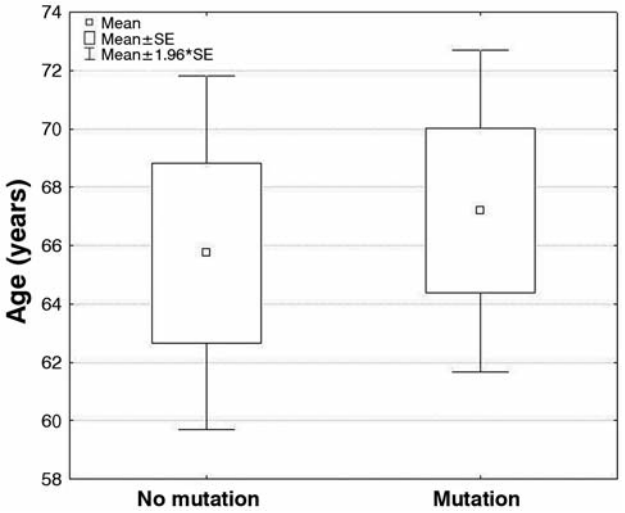

Figure 5. *Age distribution in cases of metastatic melanoma with and without BRAF V600E mutation.*

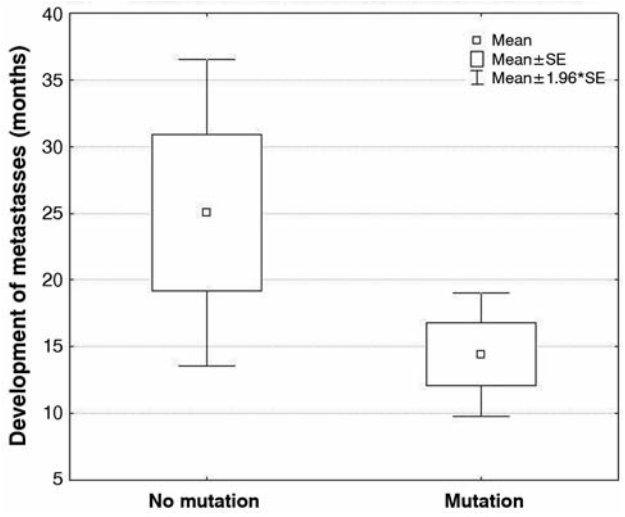

Figure 4. *Time of developing metastatic disease in patients with melanomas with and without BRAF V600E mutation.*

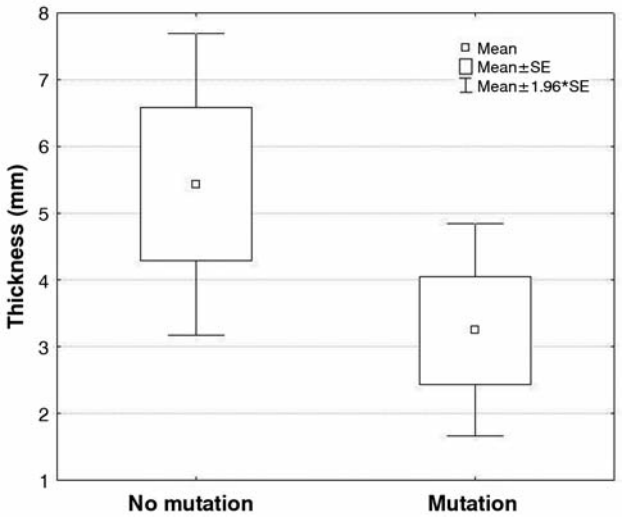

Figure 6. *Tumour thickness of metastasising primary melanomas grouped by mutated and non-mutated tumours. Growth fraction as assessed by MIB-1 in metastatic melanoma with and without BRAF V600E mutation.*

MGB probe CCATCGAGATTTCACTGTAG; FAM MGB probe CCATCGAGATTTCTCTGTAG), and 25-50 ng of the sample genomic DNA and was performed on 96-well optical PCR plates.

Each genotyping plate contained template controls without DNA, and random samples were run as duplicates to confirm the successful genotyping process. SDS version 2.0 software (Applied Biosystems) was used to analyse real-time PCR data and end-point fluorescence. Genotyping data were exported from SDS software into MS-Excel data sheets for further analysis. All immunochemical reactives were commercially available from Dakopatts (Sweden), Nordic Biosite (Sweden) and Immunotech (France). Data related to the histology of the primary tumours, as well as some clinical data, were available from a computerized file archive system. Statistical analysis was performed using Fisher's exact test, ANOVA and *t*-test. The level of significance was set at ≤0.05. Statistics and tables were made using Statistica™ software (Statsoft Scandinavia AB, Uppsala, Sweden). The study was approved by the local Ethics Committee (protocol number 2005/1331-31/4).

Results

Diagnostic immunostaining with monoclonal antibodies to the S100, vimentin and HMB-45 antigens resulted in distinct cytoplasmic staining (Figure 1). Staining with monoclonal MIB-1 antibody was strictly nuclear (Figure 2). All cases

Figure 7. *Clark's invasion level of metastasizing primary melanomas grouped by mutated and non-mutated tumours.*

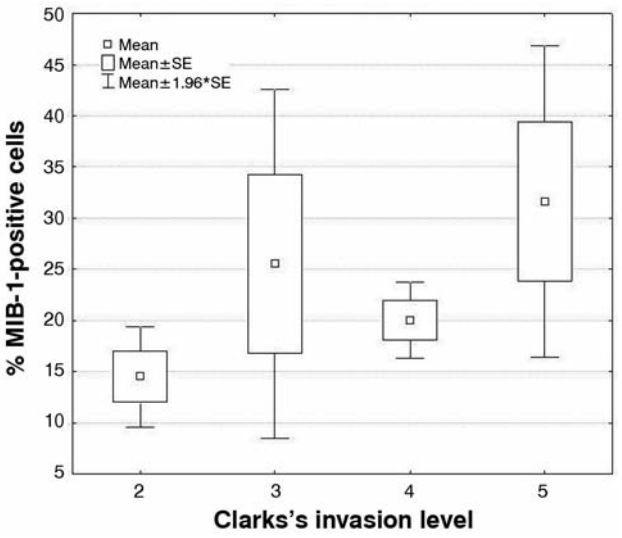

Figure 8. *Relationship between growth fraction (assessed by MIB-1) and Clark's invasion depth in melanomas.*

stained positive for S100 and vimentin. A total of 39 cases (90.6%) were positive for HMB-45 as well. In 17 (38.6%) cases, a *BRAF* V600E mutation was detected. In the group of melanomas in regions with chronic sun exposure (7 cases), only 1 case (14.28%) had a *BRAF* V600E mutation, whereas in the group without chronic sun exposure (37 cases), 15 cases (40.5%) had this type of somatic mutation *(p*=0.18820). The fraction of proliferating melanoma cells as measured by MIB-1 staining was slightly higher in the group of metastatic melanoma with mutation compared to that without mutation. The mean percentage of MIB-1-positive cells was 20.7% in non-mutated tumours and 26.2% in tumours with *BRAF* V600E mutation, respectively (*t*-value= –1.04374, *p*=0.302873, Figure 3). The distribution of mutated and non-mutated cases of metastatic melanoma within groups of different cytological cell types is shown in Table I. It can be seen from this Table that the *BRAF* V600E mutation was not restricted to any cytological type of metastatic melanoma.

None of the metastases from skeletal muscle or liver revealed the *BRAF* V600E mutation. In metastases from lymph nodes, 13 cases (46%) had the V600E mutation of the BRAF gene. From 11 cases of skin metastasis, 4 cases (36%) revealed the studied mutation.

Mutated and non-mutated cases showed a different time of development of metastatic disease, with the to metastasis of 25.0 and 14.4 for cases with and without mutation, respectively (*t*-value=1.45061; *p*=0.156620, Figure 4). The age distribution was similar in melanomas with and without mutation (Figure 5). The presence of the analysed *BRAF* V600E mutation in metastatic melanoma was correlated to the histomorphological properties of the corresponding primary tumours such as tumour thickness and Clark's invasion level. The data are summarized in Figure 6 and Figure 7. It can be seen from these Figures that both tumour thickness (*t*=1.410073; *p*=0.168802) and Clark's invasion level (*t*=2.527527; *p*=0.016990) was lower in primary tumours with *BRAF* V600E mutation compared to those without mutation. The growth fraction as assessed by the MIB-1 antibody and measured as the percentage of positive cells was compared with respect to histomorphological properties of the primary tumours. The results summarized in Figure 8 and Figure 9 show that the growth fraction of melanomas had an insignificant tendency to be higher with such progression sign as tumour invasion depth (*p*=0.269188) but not thickness (*p*=0.592970).

Discussion

Malignant melanoma is a highly aggressive malignancy, which is incurable in the majority of patients with available therapy at a metastatic stage (15-17). Thus, development of effective systemic therapy is of utmost interest and importance. Several agents targeted at different points in signalling pathways of cell proliferation and melanoma progression have been tested with very limited success. One of the most important phenomenon in the development of melanoma is the activation of the RAS-RAF-mitogen-activated protein kinase (MAPK) pathway. RAS-regulated kinases, encoded by *BRAF*, mediate cell growth and malignant transformation (18). Sorafenib is the first oral multi-kinase inhibitor to be developed that targets and inhibits Raf kinases (RAF-1, wild-type BRAF, and BRAF V600E). Sorafenib was recently demonstrated to have an anti tumour effect in metastatic renal cell carcinoma (19)

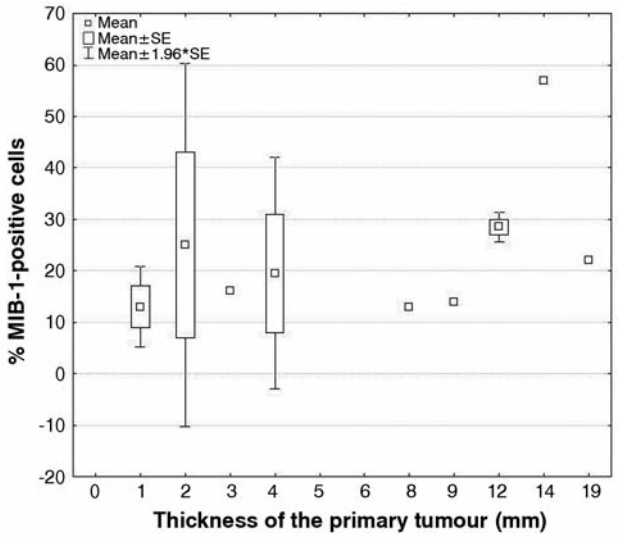

Figure 9. *Relationship between growth fraction (assessed by MIB-1) and tumour thickness in melanomas.*

and further investigation is ongoing in several tumour types such as hepatocellular carcinoma (20), leukaemia (21), prostate (22) and colorectal carcinomas (23). If the response to RAF kinase inhibition is dependent on the presence of an activated BRAF protein, it will be necessary to evaluate cases of malignant melanoma for the presence or absence of *BRAF* mutations.

In the present study, we analysed *BRAF* V600E mutation in fine-needle aspirates from metastatic melanomas. Our results show that 38.6% of metastatic melanomas possess this type of somatic mutation and that it is more often present in melanomas arisen in sites without chronic sun-induced damage (40.5% of cases) compared to that of sites chronically exposed to the sun (14.28% of cases). These results are in agreement with previous studies (24). The presence of *BRAF* V600E mutation was not correlated to any of the cytological cell types. Interestingly, the melanoma cases with a *BRAF* V600E mutation had a tendency for a shorter time for developing metastatic disease compared to those cases without mutation. Moreover, comparison of histomorphological properties of corresponding primary tumours revealed that melanomas with *BRAF* V600E tended to acquire metastatic potential at lower Clark's invasion level and lower thickness. This relationship can be seen from Figures 6 and 7. This may indicate that assessing the *BRAF* V600E mutation in melanomas can add prognostic information to the traditional markers such as tumour thickness and invasion level. Mutated tumours may thus have a more aggressive clinical behaviour because such melanomas metastasize at an earlier stage.

The presence of the *BRAF* V600E mutation was not confined to any specific localization: 13 out of 28 cases of metastatic tumours from lymph nodes and 4 out of 11 cases from skin metastases showed the mutation. None of the metastases from skeletal muscle or liver revealed the mutation. However, the number of cases in these groups was rather low.

We also analyzed the growth fraction in metastatic melanomas by MIB-1 monoclonal antibody staining. Our results show that advanced melanoma cases have a higher growth fraction than those with a less advanced stage. This observation is in agreement with our previous results (25). Moreover, the present study shows that melanomas with *BRAF* V600E mutation have a higher proliferating rate. Thus, estimation of growth fraction of malignant melanomas by MIB-1 may have additional prognostic value.

In conclusion, a proportion of melanoma cases possess a *BRAF* V600E mutation which occurs more often in sites without chronic sun exposure. Cases with *BRAF* V600E mutation might have more aggressive clinical course. Studies are needed for the evaluation of the effect of treatment with RAF-kinase inhibitors in metastatic melanomas with *BRAF* mutation.

Acknowledgements

This study was supported by funds from the Cancer Society of Stockholm (Cancerföreningen) in Stockholm and KIRT research-training coopoeration programme between Karolinska Institute and Latvian Medical Academy.

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Received October 16, 2010
Revised June 16, 2010
Accepted June 28, 2010