Lack of Association between *BRAF* Mutation and MAPK ERK Activation in Melanocytic Nevi

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The mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase signaling pathway can be activated through mutations of V-RAF murine sarcoma viral oncogene homolog B1 (*BRAF*) oncogene, frequently found in melanoma (60%), common nevi (CN) (73–82%), and atypical nevi (AN) (52–80%). MAPK activation has been reported between 0 and 22% in nevi, and 86% of primary melanoma, without any knowledge of *BRAF* mutational status. We studied the correlation of MAPK activation status, *BRAF* mutation, and B-Raf expression in CN, AN, and melanoma. Using immunohistochemistry, phosphorylated (active) MAPK and B-Raf expression was studied in 24 CN, 21 AN, and 26 primary cutaneous melanomas (PM). *BRAF* mutations at codon 600 were assessed by PCR-RFLP. Active MAPK was detected in 29% of CN, 48% of AN, and 85% of PM. *BRAF* mutation was found in 67% of CN, 62% of AN, and 58% of PM. In all, 23% of CN, 54% of AN, and 93% of PM with *BRAF* mutation have activated MAPK. All lesions expressed B-Raf. *BRAF* mutation does not seem to be sufficient to produce MAPK activation in melanocytic nevi, and it is suggested that other events are needed to induce MAPK activation, that is, B-Raf overexpression, inhibition of MAPK phosphatases, or suppression of RAF kinase inhibitors.

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INTRODUCTION

Cutaneous melanoma is the most lethal skin cancer, and its overall incidence has risen rapidly in the last decades (Jemal *et al.*, 2003). In human melanocytic proliferations, there are a number of discrete lesions, including benign nevi, dysplastic or atypical nevi (AN), and *in situ* and invasive melanomas (Clark *et al.*, 1984). Although commonly AN are considered as stable melanocytic proliferations, it is also thought that they represent both a marker of increased risk for melanoma and a potential precursor lesion (Rhodes *et al.*, 1983; Duray and Ernstoff, 1987), because many melanomas (up to 30%) seem to arise from pre-existing AN (Sober and Burstein, 1995).

Although the pathogenetic mechanisms underlying melanocytic transformation are still largely unknown, melanoma seems to be related to both environmental and genetic factors (Polsky *et al.*, 2001). Some of the key processes underlying human tumor development and progression are tumor suppressor gene inactivation, impaired DNA repair, and dominant oncogene activation (Hahn and Weinberg, 2002). In melanoma, a number of genetic abnormalities affecting a variety of these genes have been reported, including *CDKN2A* and *PTEN* inactivation (Pollock and Trent, 2000) and *RAS* and V-RAF murine sarcoma viral oncogene homolog B1 (*BRAF*) oncogene mutation (Davies *et al.*, 2002; Omholt *et al.*, 2002).

The RAS–RAF–mitogen activated protein kinase kinase/extracellular signal-regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) or mitogen-activated protein kinase (MAPK) ERK is an important pathway that mediates cellular responses to growth signals (Peyssonnaux and Eychene, 2001), and can be activated in melanoma through growth factors, and *RAS* and *BRAF* mutations. *RAS* mutation frequency (especially *N-RAS*) varies between 5 and 36% (Omholt *et al.*, 2002). *BRAF* activating mutations have been found in a high proportion of melanomas (56–66%), AN (52–80%), and common nevi (CN) (73–82%) (Davies *et al.*, 2002; Pollock *et al.*, 2003; Uribe *et al.*, 2003). *BRAF* encodes a serine/threonine kinase that transduces regulatory signals from Ras to Mek1/2. *BRAF* mutations are within the kinase domain, with a single substitution in exon 15 (T1799A) at codon 600 (V600E) accounting for 83% of *BRAF* mutations in primary melanoma and 95% in melanoma cell lines (Davies *et al.*, 2002). Mutated B-Raf proteins have elevated kinase activity, induce ERK activation, and transform NIH3T3 cells (Davies *et al.*, 2002). The importance of this MAPK pathway in melanocytic malignant transformation and melanoma progression is partially reflected in the fact that introduction of MEK activated or V600EBRAF into immortalized murine melanocytes leads to tumorigenesis *in vitro* and *in vivo* (Govindarajan *et al.*, 2003; Wellbrock *et al.*, 2004).

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Abbreviations: AN, atypical nevi; BRAF, V-RAF murine sarcoma viral oncogene homolog B1; CN, common nevi; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase

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While *BRAF* mutation is frequently found in melanomas and nevi, and is considered an early event in the pathogenesis of melanocytic lesions (Davies *et al.*, 2002; Pollock *et al.*, 2003; Uribe *et al.*, 2003; Kumar *et al.*, 2004; Miller *et al.*, 2004), MAPK ERK1/2 activation is reported only between 0 and 22% in nevi, and 54–86% of primary melanomas (Cohen *et al.*, 2002; Jorgensen *et al.*, 2003; Satyamoorthy *et al.*, 2003), suggesting that in nevi *BRAF* mutation is not sufficient to induce MAPK activation *in vivo*. Previous studies have not simultaneously analyzed *BRAF* mutation and MAPK activation in CN and AN. On the other hand, there is no information regarding B-Raf expression in human melanocytic lesions *in vivo*.

In the present study, we show that ERK1/2 is more frequently activated in AN than in CN, but there is a lack of association between *BRAF* mutation and ERK1/2 activation in melanocytic nevi. B-Raf is expressed in all melanocytic lesions analyzed.

RESULTS

Expression of activated ERK1/2 in CN, AN, and cutaneous melanoma

The antibody used in the present study detects ERK1/2 only when it is catalytically activated (phospho-ERK1/2) by phosphorylation at Thr202 and Tyr204 (Albanell *et al.*, 2001; Arbiser *et al.*, 2001). The specificity of phospho-ERK1/2 antibody was confirmed by immunostaining formalin-fixed, paraffin-embedded cytoblock preparations of the melanoma cell line SK-MEL-28 grown in cell medium with serum (Figure 1a) and the same cells in starvation, and treated with an inhibitor of MEK, U0126 (Figure 1b). Endothelial cells and lymphocytes were considered as internal positive and negative controls for each slide (Figure 1c), as used by others (Gioeli *et al.*, 1999; Albanell *et al.*, 2001). A heterogeneous cytoplasmic and/or nuclear staining pattern was observed as a positive immunostaining (Figure 1c and d). In general, ERK1/2 phosphorylation was found in only a small fraction of nevus or melanoma cells, especially in the superficial component of melanoma (Figure 1c–f) and AN (Figure 1h), and more scattered in CN (Figure 1j). Activated ERK1/2 was detected in seven of 24 CN (29%), 10 of 21 AN (48%), and 22 of 26 PM (85%) (Table 1).

To ensure that negative immunostaining was due to the lack of phosphorylated ERK1/2, 11 negative cases (four CN, four AN, and three melanomas) were stained with an antibody targeting both phosphorylated and unphosphorylated ERK1/2. Diffuse cytoplasmic and nuclear staining was seen with this antibody in the majority of normal and tumoral cells in all cases (data not shown).

Association between *BRAF* mutation at codon 600 and MAPK activation in nevi

BRAF mutations at codon 600 were found in 67% of CN, 62% of AN, and 58% of primary melanomas. *BRAF* mutational status at codon 600 was correlated with MAPK ERK1/2 activation by immunohistochemistry, finding that 23% of CN and 54% of AN with *BRAF* mutations have activated MAPK (Table 1). In contrast, 93% of melanomas with mutated *BRAF* have activated MAPK, this difference being statistically significant (Table 1). In all groups, there were tumors with activated MAPK, but without *BRAF* mutation at codon 600 (Table 1).

B-Raf expression in melanocytic lesions

The antibody used to study B-Raf expression detects mutant and wild-type protein (data not shown). B-Raf is expressed in the epidermis, sebaceous glands, fibroblasts, and endothelium in a diffuse and tenuous form, although in sweat glands it is detected in a more intense form and there is no staining in smooth muscle cells. The specificity of B-Raf antibody was confirmed by incubating with B-Raf antibody and its blocking peptide, resulting in the absence of immunostaining (Figure 1k and l). The expression of B-Raf is essentially cytoplasmic (Figure 1m), and it is expressed in all the melanocytic tumors, although the intensity of staining is variable. In general, in melanoma, there is a more diffuse and intense immunostaining (Figure 1k, m, and n); in AN it is more intense at the epidermis and junctional levels (Figure 1o); in CN, the staining is notably weaker than in melanoma, and it is preferentially seen at the epidermis or upper dermis (Figure 1p).

DISCUSSION

Activation of MAPK was found in 29% of CN, 48% of AN, and 85% of melanomas. Activated MAPK showed a similar frequency as reported previously (Cohen *et al.*, 2002). Different reports show immunohistochemical expression of activated MAPK in CN from 0 to 75% (Cohen *et al.*, 2002; Jorgensen *et al.*, 2003; Saldanha *et al.*, 2004). Cohen *et al.* (2002), using the same antibody of the present study, found MAPK activation in 22% of benign nevi with mild atypia, a percentage similar to our results.

On the other hand, 23% of CN and 54% of AN with *BRAF* mutation have activated MAPK. In contrast, 93% of melanomas have activated MAPK in the presence of mutated *BRAF*. AN lies in an intermediate position between CN and melanoma, and this is related to its potential as a melanoma precursor lesion. Nevi and melanomas with activated MAPK, but without *BRAF* mutations at codon 600, were also found. This situation can be explained by the following: (1) Ras mutation: NRAS mutations of between 5 and 36% have been detected in melanoma (Omholt *et al.*, 2002) and in a small fraction of CN (Pollock *et al.*, 2003). (2) *BRAF* mutation at codon 600 is the commonest mutation, but still about 10% of *BRAF* mutations can be found at other codons (Wan *et al.*, 2004). (3) The presence of paracrine/autocrine growth factor stimulation rather than *RAS* or *BRAF* mutations.

The absence of activation of MAPK in the presence of *BRAF* mutation, especially in CN and AN, cannot be explained by the absence of expression of B-Raf or total ERK1/2, but by the existence of additional mechanisms inducing activation of the pathway (ie, B-Raf overexpression or complementary paracrine/autocrine growth factor stimulation) (Satyamoorthy *et al.*, 2003) and/or inhibitory mechanisms that reduce the expression or activity of B-Raf or other protein(s) related to activation of ERK1/2:MAPK phosphatases

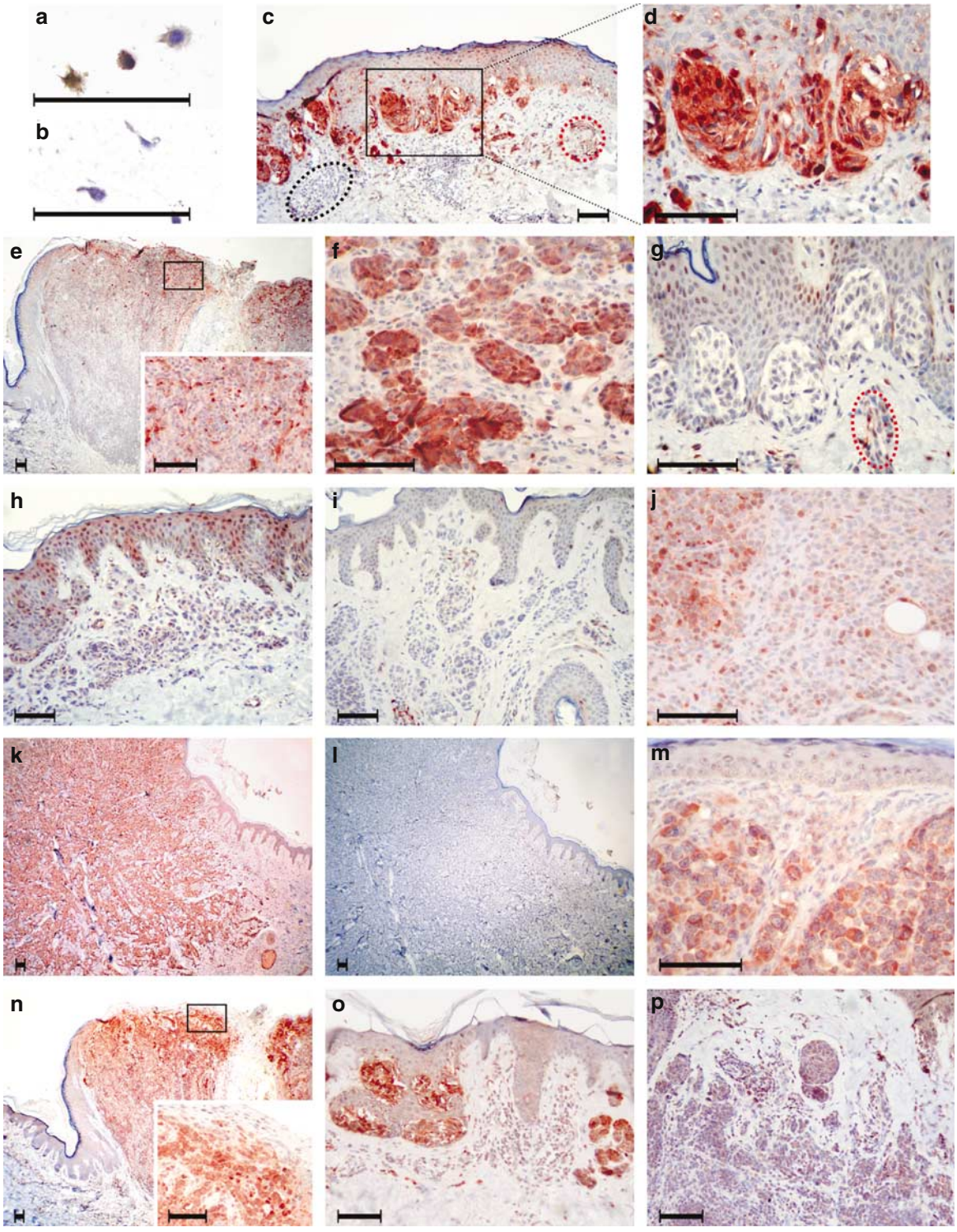

Figure 1. Phosphorylated ERK1/2 and B-Raf expression in melanocytic lesions by immunohistochemistry. Activated MAPK (**a–j**) and B-Raf (**k–p**) immunohistochemistry. (**a**) Formalin-fixed, paraffin-embedded SK-MEL-28 cells (*BRAF* V600E mutation) in culture media containing 10% of fetal bovine serum. (**b**) Cells were cultured in starvation and with a MEK inhibitor (20 μM U0126). (**c**) Melanoma with intense activated MAPK staining. Lymphocytes (black dashed oval) and endothelial cells (red dashed circle) were used as internal negative and positive controls. (**d**) The higher magnification picture shows nuclear and cytoplasmatic staining. (**e**, **f**) Melanomas with positive staining. (**g**) AN negative for activated MAPK, but endothelium reacts with the antibody (red dashed oval). (**h**) AN with positive staining for activated MAPK. CN negative (**i**) and positive (**j**) for activated ERK1/2. (**k**) PM with intense and diffuse B-Raf expression. (**l**) The same melanoma as in (**k**), but incubated with B-Raf antibody and a 10-fold excess of B-Raf blocking peptide. (**m**) Melanoma showing intense cytoplasmic staining. (**n**) Melanoma with intense expression of B-Raf. (**o**) AN with intense staining in the junctional component. (**p**) CN showing weak B-Raf expression, greater in the upper dermis. Bar=100 μm.

Table 1. Relationship between *BRAF* mutation at codon 600 and expression of activated ERK1/2 (P-ERK1/2) in nevi and melanomas

Tumor	*BRAF* mutations at codon 600	P-ERK1/2 expression		Cases with mutated *BRAF* and P-ERK1/2 expression
		Yes	No	
Common nevi (CN)	*67% (16/24)*	*29% (7/24)*		*3/16 (23%)*
Mutated *BRAF*		3	13	
Wild-type *BRAF*		4	4	
Atypical nevi (AN)	*62% (13/21)*	*48% (10/21)*		*7/13 (54%)*[1]
Mutated *BRAF*		7	6	
Wild-type *BRAF*		3	5	
Primary melanoma (PM)	*58% (15/26)*	*85% (22/26)*[2,3]		*14/15 (93%)*[4,5]
Mutated *BRAF*		14	1	
Wild-type *BRAF*		8	3	

[1]Represents $P<0.064$, between CN and AN.
[2]Represents $P<0.001$, between CN and PM.
[3]Represents $P=0.010$, between AN and PM.
[4]Represents $P<0.001$, between CN and PM.
[5]Represents $P=0.002$, between AN and PM.

(Brondello *et al.*, 1997; Arbiser, 2003) and inhibitors of RAF kinases (Schuierer *et al.*, 2004). Although practically all melanomas bearing mutated *BRAF* present activation of MAPK and only a small fraction of the nevi follows the same pattern, the existence of MAPK-independent cellular effects of *BRAF* mutation in nevi cannot be discarded (Hindley and Kolch, 2002; Ikenoue *et al.*, 2003).

In summary, we have shown that in nevi, especially in CN, *BRAF* mutation is not the only event to induce MAPK activation, as it commonly occurs in melanoma. This fact suggests the existence of still preserved control mechanisms in most of the nevi and that these are lost in melanoma.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded tissues from 24 CN (19 intradermal, four compound, and one junctional nevi), 21 AN, and 26 primary cutaneous melanomas (PM) (12 superficial spreading melanomas, five nodular melanomas, three acral lentiginous melanomas, two lentigo maligna melanomas, and four other histological variants) from 64 patients were obtained from the files of the Department of Pathology of the Catholic University School of Medicine. The experiments conducted in this study were in accordance with the Declaration of Helsinki Principles and had institutional approval.

Antibodies and other reagents

The primary antibodies used in this study were rabbit polyclonal phospho-p44/42 MAPK (Thr202/Tyr204) antibody to activated ERK1/2 (New England BioLabs Inc., Beverly, MA), rabbit polyclonal ERK1/2 antibody to total ERK1/2 (New England BioLabs Inc.), and rabbit polyclonal Raf-B antibody (SC-166, Santa Cruz Biotechnologies Santa Cruz, CA). For B-Raf immunohistochemistry, a B-Raf blocking peptide (SC-166P, Santa Cruz Biotechnologies Inc.) as a control for antibody specificity was also used (1:50 dilution). The specific Mek inhibitor U0126 (Favata *et al.*, 1998) was purchased from Promega (Madison, WI).

Immunohistochemistry

All specimens were fixed in 10% buffered neutral formalin, dehydrated, and paraffin embedded. Immunostaining was performed using 5-μm tissue sections placed on silanized glass slides. After deparaffinization in xylene and graded alcohols, epitope retrieval was performed. Target retrieval for activated ERK1/2 and total ERK1/2 was made in 10 mM EDTA buffer (pH 8) in two steps of 16 and 6 minutes, respectively, in a microwave oven at 850 W. Epitope retrieval for B-Raf was made in 10 mM EDTA buffer (pH 8) using three rounds of 7 minutes each in a microwave oven at 850 W. After epitope retrieval, endogenous peroxidase was blocked by immersing the sections in 3% hydrogen peroxide for 15 minutes. Incubations with primary antibodies were made at room temperature overnight at the following dilutions: activated ERK1/2 1:100, total ERK1/2 1:200, and B-Raf 1:500. Dilutions were made using common antibody diluent (Biogenex, San Ramon, CA). A streptavidin–biotin multilink method (StrAviGen Multilink kit; Biogenex, San Ramon, CA) using an automated staining System (Optimax I 6000, Biogenex) was used for detection of reactivity. Sections were stained with a secondary multilink antibody (1:70 dilution for 20 minutes), followed by horseradish-peroxidase-labeled streptavidin complex (1:70 dilution for 20 minutes). 3-Amino-9-ethylcarbazole was used as chromogen and applied for 10 minutes. Sections were counterstained with hematoxylin and mounted. All of the series included positive and negative controls. Positive controls were sections of a tissue specimen previously found to be positive for the antigen of interest. The negative controls consisted of sections in which the primary antibody had been excluded and replaced with common AB diluent (Biogenex Inc.). Tumors with >1% of tumor cells staining for activated ERK1/2 in the cytoplasm and/or nucleus were considered

positive (Gioeli *et al.*, 1999), and tumors with >5% of tumor cells staining for B-Raf in the cytoplasm were considered positive. For activated ERK1/2, endothelial cells and lymphocytes were used as internal positive and negative controls, respectively (Gioeli *et al.*, 1999; Albanell *et al.*, 2001). To ensure that negative immunostaining was because of a lack of phosphorylated ERK1/2, 11 negative cases were stained with a total ERK1/2 antibody. To evaluate the immunohistochemical specificity of activated ERK1/2 antibody, 20 μM MEK inhibitor U0126 (Promega, Madison, WI) in SK-Mel28 cell line was used. After treatment, cells were formalin fixed and paraffin embedded, before the immunostaining, and 3,3-diaminobenzidine was used as chromogen.

Detection of *BRAF* mutations at codon 600

BRAF mutational data, for the most part, of microdissected samples from archival paraffin-embedded tissues were available from a previous study, and for new samples the same PCR-RFLP strategy was used (Uribe *et al.*, 2003). Briefly, a 170-bp product was obtained using PCR amplification and later it was cut with *Tsp*RI. Mutated DNA was cut into two fragments (135 and 35 bp) and wild-type DNA was cut into three fragments (87, 48, and 35 bp). To test the efficacy of the restriction enzyme in each digestion, DNA from one case of a mutated melanoma sample was used as a positive control, and DNA extracted from stromal cells was used as a negative control for the mutation. At least two independent experiments were performed on each sample to assure that the analysis was reproducible.

Statistical analysis

A comparison between the expression of activated ERK1/2 and *BRAF* mutation was performed using the Fisher exact test. All of the statistical tests were conducted at the two-sided 0.05 level of significance.

CONFLICT OF INTEREST

The author states no conflict of interest.

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