

# Constitutive Mitogen-activated Protein Kinase Activation in Melanoma Is Mediated by Both BRAF Mutations and Autocrine Growth Factor Stimulation<sup>1</sup>

Kapaettu Satyamoorthy,<sup>2</sup> Gang Li,<sup>2</sup> Michelle R. Gerrero, Marcia S. Brose, Patricia Volpe, Barbara L. Weber, Patricia van Belle, David E. Elder, and Meenhard Herlyn<sup>3</sup>

Wistar Institute [K. S., G. L., M. H.]; Abramson Family Cancer Research Institute, University of Pennsylvania Cancer Center [M. R. G., M. S. B., P. V., B. L. W.]; and Anatomic Pathology, Hospital of the University of Pennsylvania [P. v. B., D. E. E.], Philadelphia, Pennsylvania 19104

## Abstract

Dysregulated activation of Ras or its downstream effectors such as mitogen-activated protein kinase kinase and ERK has been shown to play a critical role in tumorigenesis of many cancer types. However, in melanoma, activating mutations in Ras are rarely observed and are limited to N-Ras in UV-exposed cells. In this study, we identify constitutively activated ERK in almost all melanoma cell lines and in tumor tissues tested, which is in contrast to normal melanocytes and several early stage radial growth phase melanoma lines where ERK can be activated by serum or growth factors. Constitutive activation of ERK is preceded by phosphorylation of mitogen-activated protein kinase kinase and c-RAF. In all of the melanoma cell lines tested, Ras is constitutively activated without underlying mutations. On the contrary, activating mutations in the kinase domain of BRAF are present in the majority of the cell lines tested. Furthermore, ERK activation can be partially inhibited from the cell surface using inhibitors of fibroblast growth factor and hepatocyte growth factor but not interleukin 8 signaling pathways. These data suggest that melanoma growth, invasion, and metastasis are attributable to constitutively activated ERK apparently mediated by excessive growth factors through autocrine mechanisms and BRAF kinase activation.

## Introduction

Human melanocytic transformation follows distinct changes in pathological and biological aspects (1). Although the characteristics of the stepwise progression are well established (2), the precise molecular events are less understood. For normal melanocytes, paracrine growth factors, including bFGF,<sup>4</sup> HGF, stem cell factor, endothelin-1, and insulin-like growth factor-1, are essential for their proliferation, survival, and migration (3, 4). In contrast, melanoma cells predominantly use autocrine mechanisms and become autonomous (4, 5). One of the hallmarks of the growth factors in various biological systems is stimulation of their respective receptor tyrosine kinases followed by activation of MAPK Erk1/2 (6). When activated, Erks translocate to the nucleus and regulate gene expression, which leads to proliferation, differentiation, or apoptosis depending on the cell type (7). Erk1 and Erk2 are phosphorylated by MEK, which, in turn, is activated via phosphorylation by kinases of the RAF family (8). RAF kinases themselves are activated on their interaction with the GTP-bound

forms of Ras proteins (8). The roles of the Ras-RAF-MEK-Erk pathway in the regulation of melanoma growth and tumor progression are poorly understood. Ras functions have been correlated with certain human melanomas. However, activating mutations in Ras are rarely observed and are mostly limited to N-Ras in UV-exposed cells (9). Most recently, frequent BRAF activation mutations have been identified in cultured melanoma cell lines and tumor samples (10), providing new evidence that the Ras-RAF-MEK-Erk pathway plays a critical role in melanocytic tumorigenesis. In the present study we show that most melanomas have constitutively activated Erk pathway, and the signals are initiated from the cell surface through autocrine mechanisms in addition to activation mutations in BRAF. These findings suggest a causative role for MAPK activation in melanoma and identify potential targets for its control.

## Materials and Methods

**Cell Culture.** Human melanocytes from the foreskins were isolated and cultured as described (11). Melanocytes were cultured in MCDB 153 (Sigma, St. Louis, MO) supplemented with 2% chelated FBS, 2 mM glutamine (Mediatech, Herndon, VA), 20 pM cholera toxin (Sigma), 150 pM recombinant human bFGF, 100 nM endothelin-3 peptide (Peninsula, Belmont, CA), and 10 ng/ml recombinant human stem cell factor (R&D Systems, Minneapolis, MN). Human melanoma cells were isolated and maintained from clinically and histopathologically defined lesions from progressive stages of tumor development (12). These were maintained in MCDB 153 with 20% Leibovitz L-15 medium (Life Technologies, Inc., Gaithersburg, MD), 2% FBS, and 5 μg/ml insulin. For growth in protein-free medium, FBS, growth factors, and insulin were omitted as specified for cell lines. For suspension culture, melanoma cells were seeded in cell culture dish precoated with poly-HEMA (Sigma).

**Antibodies and Reagents.** Mouse anti-β-actin antibody was purchased from Sigma. Anti-p44/42 MAPK, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-MEK, and anti-phospho-c-RAF antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-pan-Ras kit was obtained from Transduction Laboratories (Lexington, KY). Replication defective adenovirus containing antisense bFGF has been reported (13).

**Mutation Analyses.** Mutations in H-Ras, K-Ras, and N-Ras at codons 12, 13, and 61 were analyzed in both the strands. Genomic DNA was extracted from melanoma cell lines after proteinase K digestion and phenol-chloroform extraction. A total of 10 ng of DNA was subjected to PCR amplification using primers as described (14) and sequencing. Mutational analysis of BRAF was performed using heteroduplex and sequence analysis. A modified heteroduplex method, CSCE, was used to screen for somatic mutations in BRAF, H-Ras, and K-Ras. The fluorescence-based CSCE technique, adapted from Rozycka *et al.* (15), is both sensitive and high-throughput. PCR primers were designed to amplify the exon plus at least 50 bp of flanking intronic sequence. Template for detection of somatic changes was 12 ng genomic DNA from the cell lines spiked with 3 ng of normal DNA to insure heteroduplex formation in case the mutation is accompanied by loss of the wild-type allele. PCR reactions were performed using standard PCR conditions with fluorescence-labeled primers. Products were denatured followed by incubation at 68°C for 1 h to allow for reannealing and the generation of heteroduplexes. The samples were then analyzed on the ABI PRISM 3100 automated capillary sequencer under semidenaturing conditions using polymer provided by ABI and optimized run

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<sup>2</sup> These authors contributed equally to this study.

<sup>3</sup> To whom requests for reprints should be addressed, at The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. Phone: (215) 898-3950; Fax: (215) 898-0980; E-mail: herlynm@wistar.upenn.edu.

<sup>4</sup> The abbreviations used are: bFGF, basic fibroblast growth factor; CSCE, conformation-sensitive capillary electrophoresis; HGF, hepatocyte growth factor; IL-8, interleukin 8; VGP, vertical growth phase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; FBS, fetal bovine serum; RGP, radial growth phase; FGF, fibroblast growth factor.

conditions. Data were captured using GeneScan to identify samples that produced a shift in peak migration relative to either the matched normal control from the same individual or a standard normal control, indicating the presence of a putative sequence variation. Amplicons, selected by the presence of a heteroduplex shift, were then sequenced directly in both the forward and reverse directions on the automated sequencer to confirm the presence of a mutation. Exons 11 and 15 were screened for BRAF mutations, exon 2 for K-Ras mutations, and exon 3 for N-Ras mutations (10).

**Western Analysis.** Cells were grown in six-well dishes except melanocytes, which were grown in 100-mm dishes. These cells were starved of serum or growth factors for indicated period of time and subsequently lysed in the extraction buffer containing 40 mM Tris (pH 7.6), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM NaVO<sub>4</sub>, 5 μg/ml of aprotinin, leupeptin, and pepstatin, respectively, and 1% Triton X-100. The extracts were spun down at 12,000 rpm for 20 min, supernatant was separated, and protein concentration was estimated using BCA assay (Pierce Chemical Co., Rockford, IL). Protein samples were loaded at 35 μg/lane, and were separated on 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The proteins were detected using appropriate primary antibody, and signal was detected using peroxidase-conjugated secondary antibody followed by development using enhanced electrochemiluminescence system (Amersham, Arlington Heights, IL).

**Ras Activation Assay.** Ras activation assay was performed as suggested by the manufacturer (Upstate Biotechnology, Waltham, MA).

**Results and Discussion**

Our previous studies have implicated that the Erk pathway plays a critical role in melanoma survival and migration (5, 16). In the present study, melanoma cell lines were analyzed for Erk1/2 status under several experimental conditions. In contrast to normal melanocytes (FOM74), in which activated Erk1/2 were not detectable under serum-free culture condition, all of the melanoma cell lines, regardless of their progression stages, clearly showed phosphorylated Erk1/2 even in the absence of serum in culture (Fig. 1A). When FBS was added to the culture medium, the phosphorylated form of Erk1/2 was induced in normal melanocytes, as well as in RGP cell lines SBcl2, WM35, WM1232, and WM1650, and in VGP cell line WM39. None of the metastatic melanoma lines responded to serum addition. This was in accordance with our earlier observation (16). The constitutive activation of Erk1/2 in melanoma remained under suspension culture condition (Fig. 1B). The phosphorylation of Erk1/2 was inhibited by PD98059, a specific MEK inhibitor, in a dosage-dependent manner (Fig. 1C), whereas it was unaffected by phosphatidylinositol 3'-kinase inhibitor wortmannin (Fig. 1D).

Erk1/2 is activated via phosphorylation by MEK, and we examined next for the presence and activation of MEK in several melanoma cell lines. Antibody against phosphorylated MEK detected consistently activated MEK in melanoma cells and was preceded by phosphorylation of c-RAF (Fig. 1E). On activation, MAPK translocates into the nucleus where many of the targets of the MAPK signaling pathway are located. These substrates include transcription factors such as Elk-1, c-Myc, c-Jun, c-Fos, and C/EBP (17). Elk is a component of the ternary complex that binds the serum response element and mediates gene activity in response to serum and growth factors (18). Phosphorylation of Elk at Ser383 by MAPK pathway is critical for its transcriptional activity. Varying degrees of constitutive Elk phosphorylation were detected in all of the melanoma cell lines tested, with metastatic 1205Lu exhibiting strongest activation (Fig. 1E).

We also examined the activation status of Ras using an immunoprecipitation assay involving immobilized RAF-1 Ras binding domain. We observed in serum-starved cells the constitutive activation of Ras in all of the melanoma cell lines examined (Fig. 1F). However, normal melanocytes did not show activation of Ras in the absence of serum (Fig. 1F).

We next examined Erk1/2 activation in melanoma tissue samples

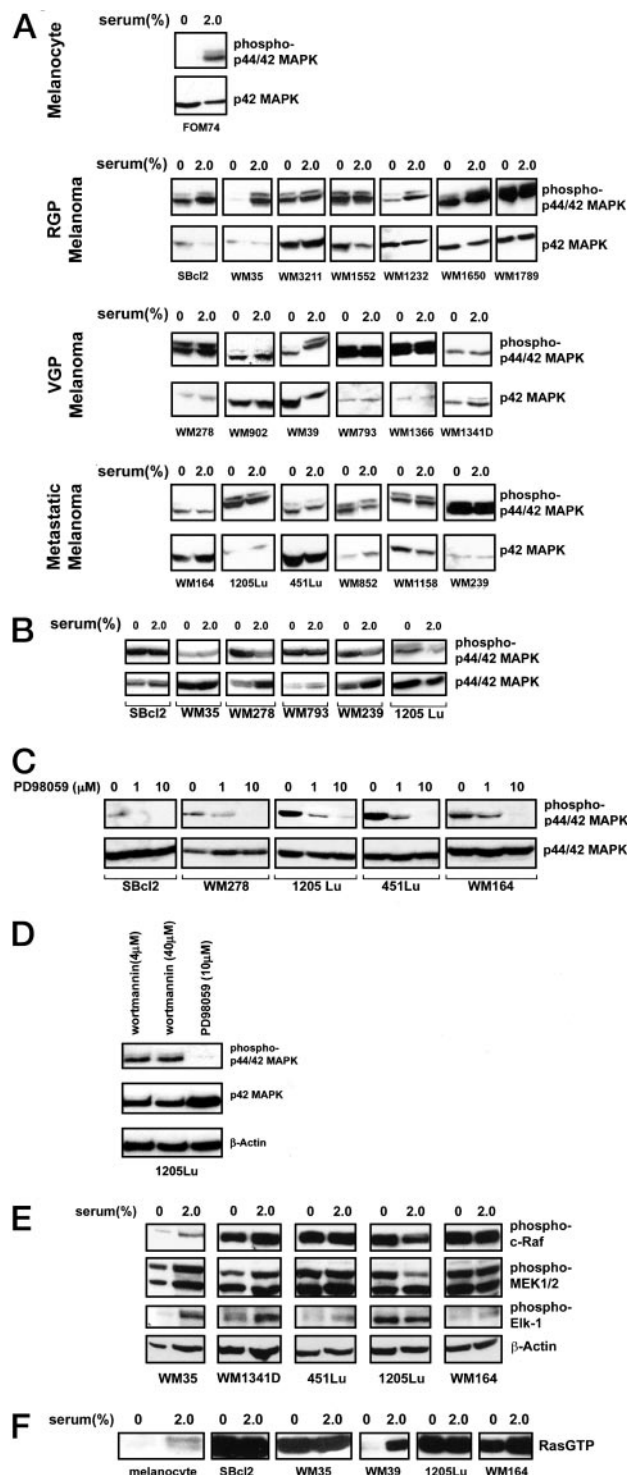


Fig. 1. Expression and status of Erk signaling pathway proteins in melanoma cells. A, melanocytes and melanoma cell lines of different progression stages were cultured in the absence (0%) or presence (2%) of FBS. The whole-cell protein extracts were separated by SDS-PAGE, and probed with anti-Erk and anti-phospho-Erk antibodies. B, Erk1/2 activation was tested in melanoma cell lines cultured in suspension in the absence and presence of serum for 24 h. C, indicated melanoma cell lines were treated with MAPK pathway-specific inhibitor PD98059 (1 μM and 10 μM) in the absence of serum for 24 h. The whole-cell extracts were subsequently analyzed for phospho-Erk and Erk levels. In the presence of 2% serum, similar patterns were observed (data not shown). D, metastatic line 1205Lu was treated with MAPK pathway-specific inhibitor PD98059 (10 μM) or phosphatidylinositol 3'-kinase pathway-specific inhibitor wortmannin (4 μM and 40 μM) for 24 h, and tested for phospho-Erk and Erk levels. E, melanoma cell lines were cultured in the absence or presence of 2% FBS, and immunodetected for levels of phospho-c-RAF, phospho-Erk, phospho-Elk-1, and internal control β-actin. F, activation of Ras in melanocytes and melanoma cells examined by immunoprecipitation using affinity tagged Ras-binding domain of RAF-1 and Western analysis using pan-Ras.

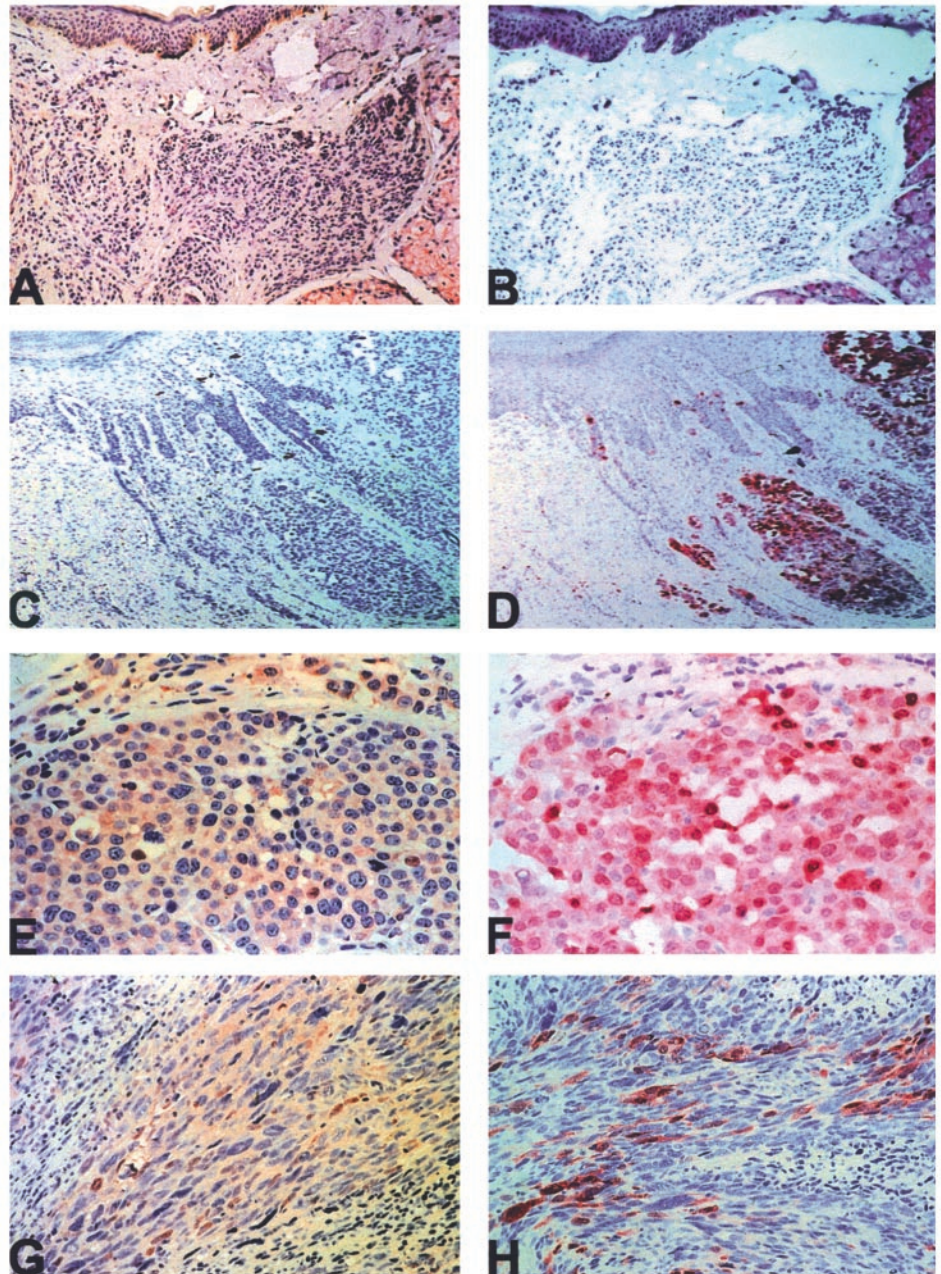


Fig. 2. Erk and phospho-Erk in melanoma tissue samples. Melanoma tissues derived from patients showing various progression levels were assayed for Erk and phospho-Erk by immunohistochemistry as described in "Materials and Methods." A, nevus stained for Erk. B, nevus stained for phospho-Erk. C, VGP control staining. D, VGP melanoma stained for phospho-Erk. E, VGP melanoma stained for Erk (high magnification). F, VGP melanoma stained for phospho-Erk (high magnification). G, metastatic melanoma stained for Erk. H, metastatic melanoma stained for phospho-Erk. (Magnification: A–D,  $\times 80$ ; E and F,  $\times 500$ ; G and H,  $\times 200$ ).

from patients harboring melanocytic lesions of defined stages (Fig. 2). Normal melanocytes and nevus cells did not exhibit phosphorylated Erk1/2 (Fig. 2, A and B), whereas VGP (Fig. 2, C–F) and metastatic melanoma (Fig. 2, G and H) tissue sections stained intensely for phosphorylated Erk.

Activating mutations in Ras have been implicated for their effects on Ras signaling pathway proteins. We examined the mutation status of Ras in all of the cell lines and did not find any activating mutations in N-Ras, K-Ras, or H-Ras (Table 1). However, the cell lines examined demonstrated activating mutations in BRAF (Table 1; Ref. 10), suggesting that RAF mutations may be, in part, responsible for the activation of Ras and subsequently Erk 1/2 in melanomas.

Besides RAF mutation, activation of Erk1/2 could be mediated through other pathways. This is hinted by the observation that, in two cell lines (SBcl2 and WM35) without BRAF mutation (Table 1), Erk1/2 was still constitutively activated (Fig. 1A), although to a less degree than most BRAF mutation-containing melanoma

cells. Cell surface signaling by growth factor receptors through autocrine mechanisms is well established in melanoma (1, 3, 5). Metastatic melanoma cell line 1205Lu was treated with adenovirus expressing antisense bFGF, neutralizing antibodies to HGF and IL-8. A dramatic inhibition of Erk1/2 activation was observed when the effects of bFGF or HGF were neutralized (Fig. 3). Anti-IL-8 antibody was marginally effective in inhibiting phosphorylation of Erk1/2 (Fig. 3). These data indicate that signaling through cell surface proteins is critical for maintaining constitutive activation of Erk1/2 in melanoma cells.

In this study we examined the expression and activation of Erk1/2 in a panel of melanoma cell lines and tumors of various stages of tumor progression. We show that Erk1/2 is constitutively activated in human melanoma cell lines and tumors in a progression stage-dependent manner. This constitutive activation is preceded by activation of MEK, RAF, and Ras proteins. We demonstrate that neutralizing cell surface signaling from FGF receptor or HGF receptor (c-Met) leads to

inhibition of Erk1/2 activation. In addition, BRAF activating mutations have been identified in most of the melanoma cell lines. These results suggest that multiple mechanisms underlie the constitutive activation of Erk1/2 in melanoma.

Aggressive melanoma cells can survive in the absence of exogenous growth stimulation by serum through several autocrine mechanisms. They express FGF receptor 1 and bFGF, forming an autocrine loop (19), and inhibition of this loop inhibited Erk1/2 phosphorylation (20). Melanoma cells also express c-Met and secrete its ligand HGF (5). We have shown before that HGF induces Erk1/2 phosphorylation through activation of its receptor, c-Met, which can be inhibited by neutralizing antibody against HGF (5). Therefore, HGF and FGF-2 may act in an additive fashion to stimulate Erk1/2 activation and, hence, proliferation and migration of melanoma cells. However, melanoma in a tissue context may have additional support from paracrine mechanisms such as insulin-like growth factor-1 and platelet-derived growth factor (16). Because several growth factors may converge at the Ras signaling pathway, the cumulative strength and duration of the activation signal may play an important role in melanoma progression and maintenance.

Ras (H-Ras, K-Ras, and N-Ras) gene mutations are detected frequently in human cancers. These genes are converted to active oncogenes by point mutations occurring in codon 12, 13, or 61 (8). We analyzed mutations of these codons in a number of melanoma cell lines using CSCE and PCR sequencing method. Sequence analysis of Ras genes did not indicate any activating mutations in the codons associated frequently with tumor. However, most of the cell lines demonstrated activating mutations in the kinase domain of BRAF. A consequence of this mutation is that BRAF gains the ability to interact with MEK and several other signaling molecules without the prerequisite of being phosphorylated (10). Independent activation of BRAF without the involvement of Ras has also been reported (6). In this instance, a parallel pathway for activation of BRAF through protein kinase A has been proposed. However, the extent of participation of this pathway in melanoma is not clear.

In conclusion, we have demonstrated that in melanoma cell lines and melanoma tumor tissues the Erk1/2 pathway is constitutively active as a consequence of autocrine growth factor stimulation and activating mutations in the BRAF gene. Identification and understanding of precise cellular events, which precede these two independent

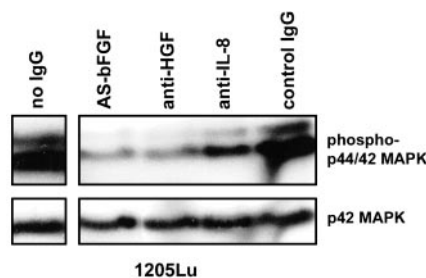


Fig. 3. Effect of growth factor-induced stimulation on Erk activation. Autocrine growth factor stimulations were inhibited by neutralizing antibodies or antisense strategy. For antibody neutralization, 1205Lu cells were serum starved for 24 h and then treated with indicated antibodies for 24 h. For antisense inhibition, 1205Lu cells were transfected with adenovirus harboring antisense bFGF sequence and cultured for 48 h. Cell extracts were analyzed by Western analysis using phospho-Erk and Erk antibodies.

mechanisms, may provide clues to critical pathways necessary for melanoma progression.

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Table 1 Ras and BRAF mutations in melanoma cell lines

Cell line <sup>a</sup>	Stage	BRAF				
		N-Ras <sup>b</sup>	H-Ras <sup>b</sup>	K-Ras <sup>b</sup>	(exon 11) <sup>c</sup>	BRAF (exon 15) <sup>c</sup>
FOM74	Normal	– <sup>d</sup>	–	–	–	–
SBcl2	RGP	–	–	–	–	–
WM1789	RGP	–	–	–	–	A1798G(K600E)
WM35	RGP	–	–	–	–	–
WM793	VGP	–	–	–	–	T1796A(V599E)
WM902B	VGP	–	–	–	–	T1796A(V599E)
WM39	VGP	–	–	–	–	T1796A(V599E)
WM75	VGP	–	–	–	–	T1796A(V599E)
WM278	VGP	–	–	–	–	T1796A(V599E)
WM1361A	VGP	–	–	–	–	–
WM1361B	VGP	–	–	–	–	T1796A(V599E)
WM1361C	Metastatic	–	–	–	–	T1796A(V599E)
WM983A	VGP	–	–	–	–	T1796A(V599E)
WM983B	Metastatic	–	–	–	–	T1796A(V599E)
WM1158	Metastatic	–	–	–	–	T1796A(V599E)
WM9	Metastatic	–	–	–	–	T1796A(V599E)
WM1617	Metastatic	–	–	–	–	T1796A(V599E)
1205Lu	Metastatic	–	–	–	–	T1796A(V599E)
451Lu	Metastatic	–	–	–	–	T1796A(V599E)

<sup>a</sup> For more information about the cell lines, see Refs. 11 and 12.

<sup>b</sup> Exons 1 and 2 of N-ras, H-Ras, and K-Ras were amplified by genomic PCR and sequenced.

<sup>c</sup> Exons were screened by CSCE followed by sequencing.

<sup>d</sup> –, no mutation detected.

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