Reduced Heterochromatin Protein 1-beta (HP1β) Expression is Correlated with Increased Invasive Activity in Human Melanoma Cells

KEISUKE NISHIMURA[2], YOSHIFUMI S. HIROKAWA[1], HITOSHI MIZUTANI[2] and TAIZO SHIRAISHI[1]

[1]Department of Pathologic Oncology, Institute of Molecular and Experimental Medicine and
[2]Department of Dermatology, Faculty of Medicine,
Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan

Abstract. *Heterochromatin protein 1 (HP1) is associated with heterochromatin formation and the regulation of gene expression. In this study, we demonstrated that decreased HP1β, but not HP1α, mRNA and protein expression, correlates with invasive potential in five human melanoma cell lines, and we used immunohistochemistry to confirm that HP1β expression is suppressed during melanoma progression. HP1β levels are decreased in ^{V600E}B-RAF-transformed mouse melanocytes, suggesting that HP1β-mediated suppressive mechanisms correlate with melanoma oncogenesis. Expression of microphthalmia associated-transcription factor (MITF), an important melanocyte differentiation factor, is reduced in melanoma, which is correlated with poor prognosis. In CRL1579, SK-MEL-28 and HMV-II human melanoma cells in which HP1β expression is reduced by RNAi, MITF RNA levels and invasiveness activities are differentially altered and are not correlated with each other. Our findings indicate that the ^{V600E}B-RAF mutation induces HP1β down-regulation, which causes epigenetic gene regulation associated with melanoma progression.*

Heterochromatin protein 1 (HP1) is associated with heterochromatin formation and plays many important roles in the regulation of gene expression. In *Drosophila melanogaster*, HP1 participates in a phenomenon known as position effect variegation, in which heritable gene suppression is caused by abnormal translocation in the proximity of heterochromatin (1-3). Moreover, targeting of HP1 to a euchromatic promoter can induce a gene-silenced state in mammals that is similar to variegation in *Drosophila* (4). Overexpression of HP1 has been shown to cause a reduction in gene expression (5, 6); HP1 is known to associate with Krüppel-associated box (KRAB) zinc finger proteins, resulting in reduced gene expression (7, 8). Thus, HP1 is considered to function during heterochromatin-associated gene expression regulation.

HP1 has the ability to interact with many partners, including DNA, RNA, and various nuclear proteins that participate in the formation and maintenance of heterochromatin (9, 10). It can bind RNA *in vitro* (11) and it has also been shown to interact with DNA and chromatin (12, 13). In addition, some reports have suggested that binding of HP1 to methylated lysine 9 of histone H3 (H3-K9) is essential for the recruitment of HP1 to heterochromatin (14-16). Binding of HP1 to these various cellular components may enable it to play an important role as a structural adaptor for heterochromatin maintenance (10).

HP1 is a highly conserved protein that has homologues in a broad spectrum of species, from *S. pombe* to mammals. Human HP1 has three isoforms: HP1α, β and γ (17). HP1 proteins are primarily located at centromeric heterochromatin, although HP1β and γ have also been shown to be associated with euchromatic regions (18, 19). HP1α can dimerize with either itself or with HP1β (20), and its targeting causes the aggregation of endogenous HP1β to chromatin (21).

HP1α is down-regulated in highly invasive/metastatic breast cancer cells in comparison to poorly invasive/non-metastatic breast cancer cells. In addition, transfection of the HP1α gene into breast cancer cells has been shown to decrease their invasive abilities *in vitro*. In concordance with this observation, HP1α expression has also been shown to be diminished in sections from various metastatic tissues in breast cancer patients (22).

Mutated forms of *RAS* and *RAF* function as oncogenes in melanoma (23-25). The RAS-RAF-MEK-ERK signaling cascade is thought to play an important role in initiation and progression of melanoma by activating a number of proteins,

Correspondence to: Yoshifumi S. Hirokawa, Department of Pathologic Oncology, Institute of Molecular and Experimental Medicine Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Japan. Tel: +81-59-232-2864, Fax: +81-59-231-5210, e-mail: ultray2k@clin.medic.mie-u.ac.jp

Key Words: Heterochromatin protein 1 beta (HP1β), ^{V600E}B-RAF mutation, microphthalmia associated-transcription factor (MITF), malignant melanoma, invasion, metastasis, RNAi.

including NF-κB, phosphatidylinositol 3'-kinases and microphthalmia-associated transcription factor (MITF) (26). MITF plays an important role during melanocyte differentiation (27) and increased MITF expression has been shown to exert antiproliferative effects in melanoma (26). Moreover, reduced MITF expression is correlated with bad prognosis in melanoma (28).

B-RAF is mutated in nearly 70% of cutaneous melanomas (29-31), and almost 80% of nevi have *B-RAF* mutations (30, 32). However, oncogenic *B-RAF* mutations may not be sufficient to initiate melanoma.

In this study, we used immunohistochemistry to demonstrate that HP1β expression is decreased in both invasive and metastatic human melanoma. We also showed that HP1β expression is reduced in ^{V600E}*B-RAF* transfected melanoma cells compared to cells transfected with wild type *B-RAF*. Recently, RNA interference (RNAi) has been shown to be a highly effective method for silencing specific genes, not only in *C. elegans* and *Drosophila,* but also in mammalian genomes (33-36). In the three melanoma cell lines tested, following an RNAi-induced reduction in HP1β expression, invasive properties and MITF RNA expression were dramatically altered and were not always correlated with each other. These results suggest that the oncogenic ^{V600E}*B-RAF* mutation causes various epigenetic gene expression changes related to melanoma invasion and progression by decreasing HP1β expression levels.

Materials and Methods

Cells and culture conditions. The human melanoma cell lines CRL1579, G361, HMV-I, HMV-II and SK-MEL-28 were supplied by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan. These cell lines were routinely cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA, USA) at 37˚C in an atmosphere of 5% CO_2 in air. Mouse melanocytes transformed with ^{WT}*B-RAF* (B2), ^{V600E}*B-RAF* (VE16) and ^{G12V}*RAS* (LTRras) have been described previously (25). ^{WT}*B-RAF* (B2) cells were cultured in RPMI 1640 (Sigma) with 10% fetal bovine serum (Gibco/Invitrogen) supplemented with 200 nM TPA (Sigma) and 0.05 μg/ml cholera toxin (Sigma) at 37˚C in an atmosphere of 5% CO_2 in air. ^{V600E}*B-RAF* (VE16) and ^{G12V}*RAS* (LTRras) lines were cultured in RPMI 1640 (Sigma) with 10% fetal bovine serum (Gibco/Invitrogen) at 37˚C in an atmosphere of 5% CO_2 in air.

RNA isolation and RT-PCR. Total RNA was isolated from melanoma cells using an RNA extraction kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was performed using the Superscript II RNase Reverse Transcriptase kit according to the manufacturer's protocol (Invitrogen). Non-RT-PCR was performed simultaneously as an internal control. cDNA synthesis was performed at 42˚C for 50 min. PCR conditions were as follows: 40 cycles of denaturation (95˚C for 15 sec), annealing (HP1α, 53˚C; HP1γ, 55˚C; MITF, 57˚C; and GAPDH, 57˚C for 15 sec), extension (72˚C for 30 sec) and 1 cycle of final extension (72˚C for 7 min). The PCR product sizes for HP1α, γ, MITF and GAPDH were 185, 192, 232 and 229 bp, respectively. The following HP1-specific primers were used for PCR: HP1α forward primer, 5'-AGACCACACCAGAAACCCTG-3'; HP1α reverse primer, 5'-GAATTTTGGTTTCTTGCTTTGG-3'; HP1γ forward primer, 5'-CAACCCCCAAGTAGAGAGCG-3'; HP1γ reverse primer, 5'-TGGAAAAGACATTGGTCCCC-3'; MITF forward primer, 5'-TGCTTCCTTTCTTGATTCGT-3'; MITF reverse primer, 5'-AGCTAAAGTCTGTGGTGAAT-3'; and GAPDH forward primer, 5'-GAAGGTGAAGGTCGGAGTC-3'; GAPDH reverse primer, 5'-CTGGAAGATGGTGATGGGATTTCC-3'. PCR products were separated on a 1.5% TBE agarose gel, stained by ethidium bromide and visualized under UV light.

Western-blot analysis. Melanoma cells were harvested in a lysis buffer (RIPA buffer; 10 mM Tris-HCl [pH 8.0], 1% NP-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, double-distilled water, protease inhibitor) and incubated on ice for 10 min. Samples were then sonicated. After an additional 10-min incubation on ice, cell debris was removed by centrifugation at 15,000 xg for 5 min and the supernatant was collected. Protein concentrations were measured with a UV mini 1240 (SHIMADZU, Kyoto, Japan). Protein samples (20 μg) were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membranes (Hybond-ECL, AmershamBiosciences, Chalfont St. Giles, UK), and blocked with 0.1% Tween 20 and 5% skimmed milk in PBS for 30 min at room temperature. Membranes were incubated with anti-HP1β antibody (Upstate/Millipore, Billerica, MA, USA) for 3 h and washed three times with 0.1% Tween 20 in PBS. Protein G HRP (BIO-RAD, Hercules, CA, USA) was used to detect bound antibodies that were visualized by exposure to film.

Preparation and transfection of HP1β-targeted RNAi. 25-nt RNAs were obtained from Invitrogen (Stealth™ RNAi). The RNAi sequences targeting HP1β (GenBank accession No. NM_006807) corresponded to the region 117-141 nt. CRL1579 cells were cultured at a concentration of $1x10^5$ cells for 48 h before transfection in RPMI 1640 with 10% fetal bovine serum in a 6-cm dish. Transfection of HP1β-targeted RNAi was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) and 200 pmol RNAi per 6-cm dish according to the manufacturer's protocol. Fifty-seven hours after RNAi transfection, total RNA or total protein were isolated for RT-PCR or Western-blotting, or cells were resuspended to perform the Matrigel invasion assay.

Matrigel invasion assay. Invasive properties of melanoma cell lines were analyzed using the BD BioCoat™ Matrigel™ Invasion Chamber according to the manufacturer's protocol (pore size, 8.0 μm; BD Biosciences, Franklin Lakes, NJ, USA). Melanoma cells were suspended in the upper chamber at a concentration of $2x10^4$ cells/well in 0.1 ml RPMI 1640 with 0.1% bovine serum albumin, and 0.7 ml RPMI 1640 with 10% fetal bovine serum was added to the lower chamber. Cells were incubated in a 5%-CO_2 atmosphere at 37˚C for 22 h. After incubation, the non-invading cells were removed from the upper side of the filter by scrubbing with a cotton swab. The membrane was fixed with 100% methanol and stained with Giemsa. The invading melanoma cells were counted under a light microscope at x100 magnification. Invasive potential was defined as the mean number of invasive cells in all fields of triplicate membranes.

Immunohistochemistry. Sections (4-μm-thick) were cut from formalin-fixed, paraffin-embedded blocks of human melanoma

tissues. After deparaffinization and rehydration, the sections were incubated in 0.01 M sodium citrate buffer (pH 6.0) for 10 min at 100˚C. The VENTANA EX system (VENTANA, Tucson, AZ, USA) was used to stain the sections with the Alkaline Phosphatase Enhanced Detection kit (VENTANA), Hematoxylin Counterstain (VENTANA), Blueing Reagent (VENTANA) and anti-HP1β antibody (Upstate/Millipore) according to the manufacturers' specifications. The sections were analyzed for the percentage of stained cells and the staining intensity, graded from 1+ (lowest) to 3+ (highest), which are indicative of HP1β immunoreactivity.

Ethical consideration. Written informed consent was obtained from each patient and permission for this study was obtained from the Ethics Board of Mie University Graduate School of Medicine, Japan.

Results

Comparison between invasive potential, HP1 mRNA and protein expression levels, and mRNA expression of MITF in melanoma cell lines. As shown in Figure 1A, HP1β protein expression levels varied in the five melanoma cell lines tested. In particular, SK-MEL-28 cells expressed low levels of HP1β protein. However, there were no robust differences in HP1α and HP1γ mRNA expression levels between the different cell lines (Figure 1B). To compare HP1β protein expression levels with invasive potential, the Matrigel Invasion Assay was performed using the BD BioCoat™ Matrigel™ Invasion Chamber, as shown in Figure 1C and 1D. SK-MEL-28 melanoma cells were more invasive than CRL1579 melanoma cells which showed higher HP1β protein expression levels (Figure 1C). SK-MEL-28 melanoma cells also appeared to be more invasive than the other melanoma cell lines with higher HP1β protein expression levels (Figure 1D). Together, these results suggest that lower HP1β protein expression may be associated with invasive potential in melanoma cell lines. Decreased expression of MITF, a regulator of melanocyte differentiation (27), is correlated with bad prognosis in melanoma (28). The most invasive cell line, SK-MEL-28, showed the lowest MITF mRNA expression level, while the HMV-II line, which has weak invasive properties, displayed the highest MITF level. This result indicated that the higher the MITF expression, the lower the invasive potential of the melanoma cells examined (Figure 1E).

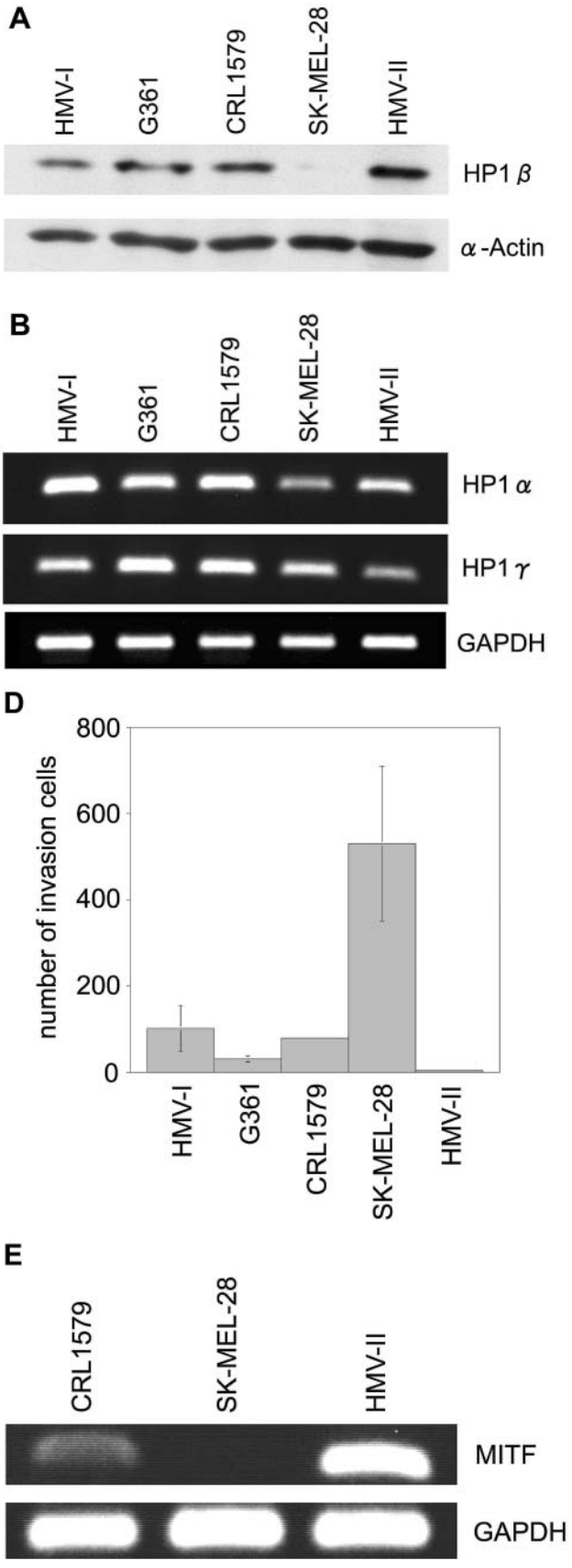
Immunohistochemical analysis of HP1β expression in human melanoma tissues. To compare the expression of HP1β with invasive and metastatic properties, immunohistochemistry was performed using an HP1β antibody in various human melanoma tissues. Formalin-fixed, paraffin-embedded human melanoma tissues (five cases of Clark's level II, eight of Clark's level IV, two lymph node metastasis and four skin metastasis) were analyzed for both the intensity of antibody staining and the percentage of HP1β-positive melanoma cells (Figure 2). Melanoma tissues from Clark's level II lesions included >70% HP1β-positive cells, and the intensity of staining was as high as 3+ (see Materials and Methods section for description of staining intensities). Out of eight invasive lesions (Clark's level IV), all showed <70% HP1β-positive staining, four indicated <5% HP1β-positive staining and all exhibited a staining intensity <2+. Furthermore, in two lymph node-metastatic cases, both lesions displayed <10% HP1β-positive staining, and one of these had a staining intensity of 1+. In contrast, in four skin metastatic cases, two patients showed <10% HP1β-positive staining along with a staining intensity of 1+. In addition, as the disease stage progressed, the percentage of HP1β-positive cells in metastatic lesions (Clark's IV ≥lymph node metastasis ≥skin metastasis) and in tissue that was resected at a later time was progressively reduced. Altogether, our results show that reduced HP1β expression is correlated with human melanoma progression.

HP1β expression levels are reduced in the ^{V600E}B-RAF-transfected mouse melan-a cell line. As shown in Figure 3, Western-blot analysis was performed with total protein from the ^{WT}B-RAF-expressing clone B2, the ^{V600E}B-RAF-expressing clone VE16 and oncogenic RAS-expressing LTRras cells to compare differences in HP1β protein expression levels. HP1β expression was lowest in the ^{V600E}B-RAF-expressing clone and highest in the oncogenic *RAS*-transfected clone.

Down-regulation of HP1β using RNAi changes, invasive activity and mRNA expression of MITF in three human melanoma cell lines. To examine the effect of HP1β down-regulation on invasiveness and MITF mRNA expression, we performed transfection with HP1β-targeted RNAi in the CRL1579, SK-MEL-28, and HMV-II melanoma cell lines. In RNAi-transfected CRL1579 cells, there were approximately 2.4 times more invading cells than in control cells ($p=0.03$; Figure 4A). In contrast, there were about 2-fold fewer invading cells in RNAi-transfected SK-MEL-28 cells than in control cells ($p<0.001$). In addition, RNAi-transfected cells also reduced invasive activity to a lesser degree in the HMV-II cell line compared to control cells ($p=0.04$). Furthermore, mRNA expression of MITF, the inhibitor of melanoma progression, was increased in CRL1579 and HMV-II cells compared to control cells. In contrast, SK-MEL-28 cells showed a reduction in MITF mRNA expression compared to control cells (Figure 4B). These results demonstrate that reduction of HP1β affects MITF gene regulation in human melanoma cell lines. Nevertheless, in melanoma, simple down-regulation of HP1β is not directly correlated with increased invasiveness.

Discussion

In this study, we showed that decreased HP1β expression is associated with high invasiveness in five human melanoma cell lines *in vitro*. The results of our immunohistochemical analysis



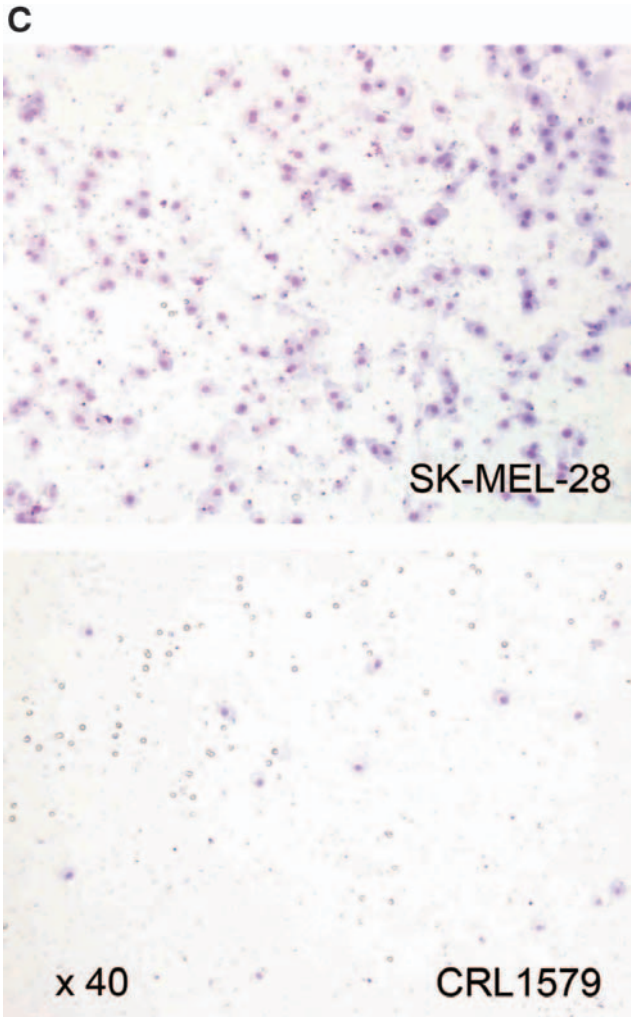


Figure 1. *A. Western-blot analysis of HP1β expression in five human melanoma cell lines. Expression of HP1β protein is lowest in SK-MEL-28, which is the most invasive line (see Figure 1D). B. HP1α and HP1γ RT-PCR analysis. There were no robust differences between HP1α and HP1γ mRNA expression in the five melanoma cell lines. C. Matrigel Invasion Assay with the BD BioCoat™ Matrigel™ Invasion Chamber. Chamber images in SK-MEL-28 and CRL1579 melanoma cell lines are shown. SK-MEL-28 melanoma cells, which express lower levels of HP1β, are more invasive than CRL1579 cells. D. Invasive properties of melanoma cell lines. The Matrigel Invasion Assay was performed in five melanoma cell lines. Invading melanoma cells were counted under a light microscope at x100 magnification. Invasive potential was defined as the mean number of invading cells in all fields of triplicate membranes. E. RT-PCR of Microphthalmia-associated transcriptional factor (MITF) expression in three human melanoma cell lines, CRL1579, SK-MEL-28, and HMV-II. The lowest level of MITF was observed in SK-MEL-28 cells, which have aggressive invasion activity, and the highest MITF levels were observed in HMV-II cells, which are associated with a lower degree of invasiveness. Reduced expression of MITF was also seen in CRL1579 cells (see Figure 1D). The MITF sequence was confirmed following gel purification of PCR product bands.*

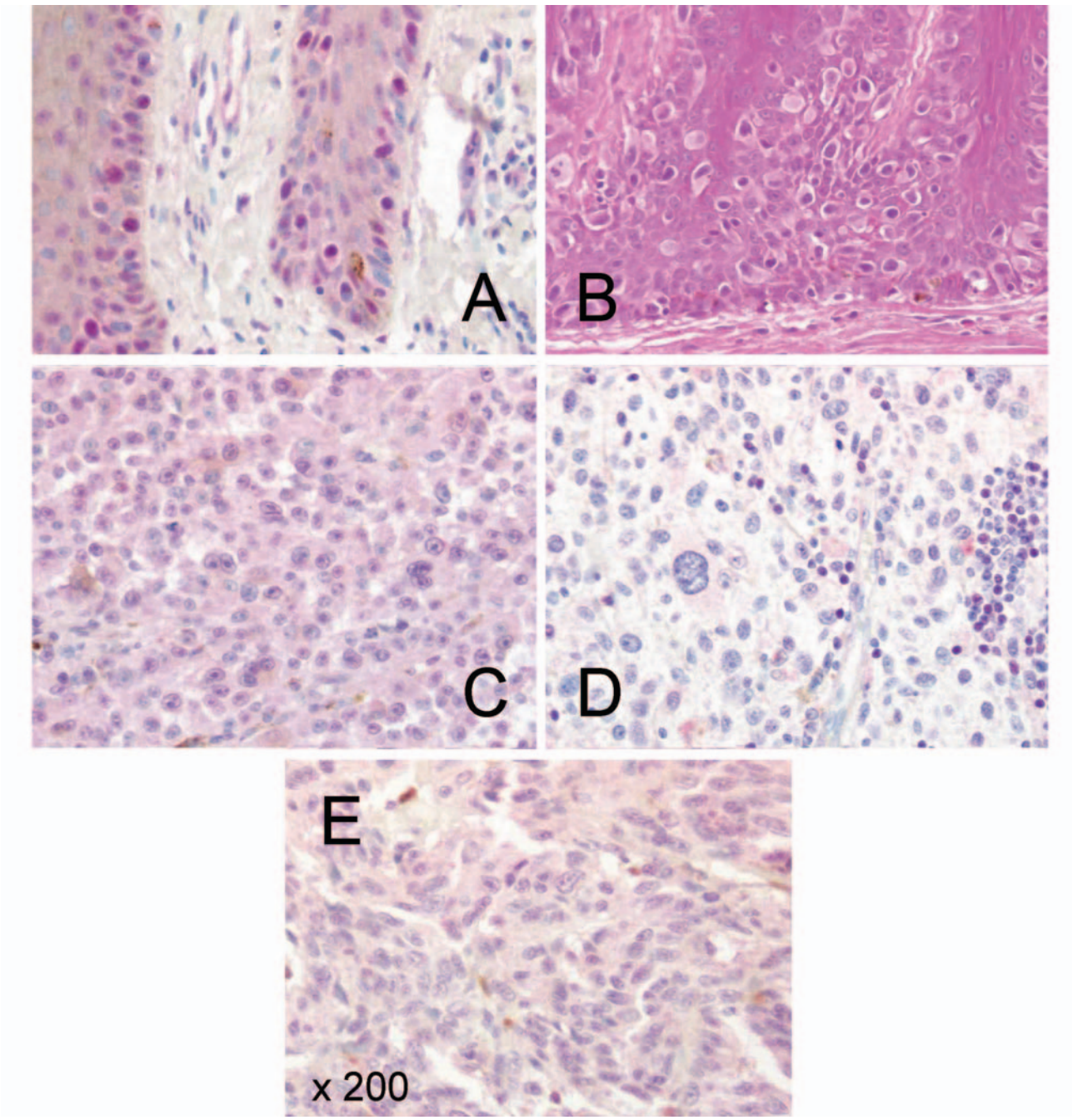


Figure 2. *HP1β immunohistochemistry in melanoma tissues. These images show HP1β expression in formalin-fixed, paraffin-embedded sections from various melanoma lesions, including Clark's level II (A), Clark's level IV (C), lymph node metastasis (D), and skin metastasis (E). HE staining was also performed in sections from the same melanoma tissues: Clark's level II (B). Reduction of HP1β was associated with melanoma cell invasion and metastasis, as shown in (C), (D), and (E). Note positive staining in keratinocytes or lymphocytes as internal controls.*

of HP1β expression in human melanoma tissue sections were consistent with these findings. As reported in the literature, overexpression of HP1α or β induces changes in the transcriptional activity of some genes, including nuclear receptor subfamily 1 group H member 4, asparagine synthetase, isoleucine t-RNA synthetase and zinc finger protein 6 (37); furthermore, overexpression of HP1α or β is associated with a reduction of cell growth activity and increased sensitivity to cell death by ionizing radiation (38). Taken together, these observations suggest that genes

Figure 3. *Comparison of HP1β protein expression levels in the ^{WT}B-RAF-expressing clone B2, the ^{V600E}B-RAF-expressing clone VE16, and oncogenic RAS-expressing (LTRras) melanoma cells. Decreased HP1β expression was observed in ^{V600E}B-RAF-expressing cells.*

required for melanoma invasion that are usually silenced or repressed by HP1β may be up-regulated *via* a decrease in HP1β levels, leading to increased melanoma invasion.

In this study, although down-regulation of HP1β expression was correlated with tumor invasion, as represented by Clark's level, Breslow's depth and degree of lymph node metastasis, there was differential expression of HP1β, particularly in Clark's level IV and skin metastasis tissues. To explain these results, we propose the following hypothesis: various genes involved in melanoma progression may be regulated through epigenetic gene silencing by HP1β during melanoma progression. Furthermore, compared with the sections of Clark's level IV and lymph node or skin metastatic tissues, both the percentage and intensity of cell staining were decreased in metastatic lesions or in those isolated at later time-points from the same patient. We interpreted this result by suggesting that melanoma cells with lower HP1β expression are more likely to be selected as metastatic clones. An alternative hypothesis is that epigenetic regulation of different genes is related both to the degree of invasiveness, as well as metastasis. Two cases demonstrated relatively high expression (≥40%) of HP1β in skin metastatic tissue, thereby supporting this second hypothesis, as skin metastasis ordinarily occurs after lymph node metastasis.

On the other hand, RNAi-induced HP1β reduction alone was not correlated with changes in invasiveness and was also not directly associated with down-regulation of MITF mRNA expression in two out of the three melanoma cell lines examined. These results suggest that decreased HP1β levels may alter epigenetic gene expression differently in each melanoma cell line. Differential changes in invasiveness may occur through haphazard changes in gene expression, and both active and inactive genes regulated by HP1β may be different in each of the melanoma cell lines tested. The mechanism through which HP1β functions in epigenetic gene regulation has, therefore, not yet been completely unveiled.

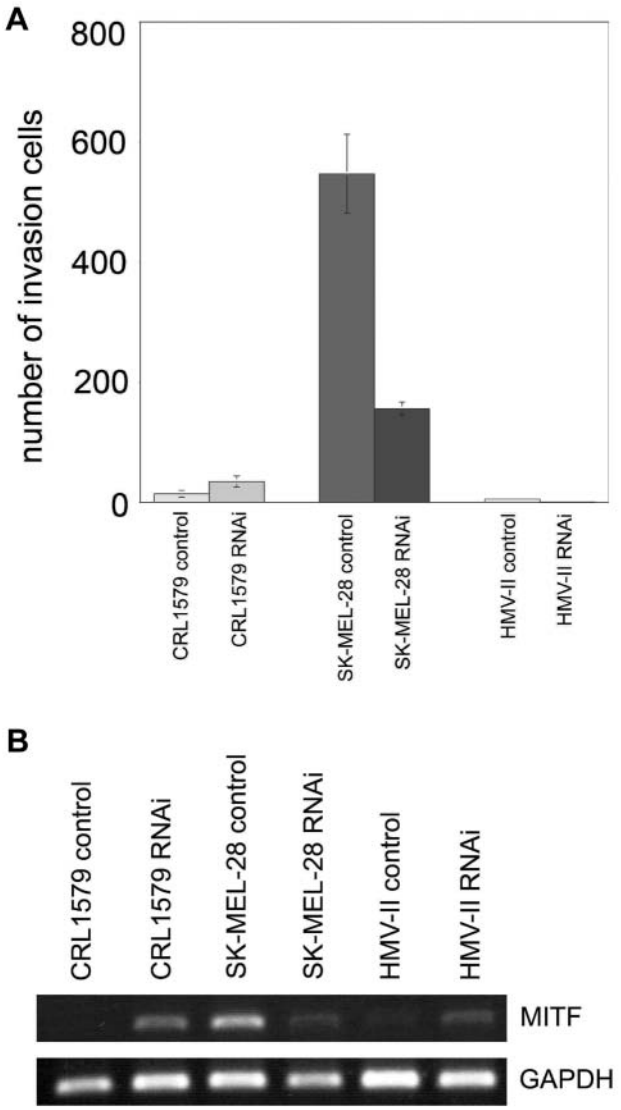


Figure 4. *A. Matrigel Invasion Assay in CRL1579, SK-MEL-28, and HMV-II cells following RNAi treatment. Invading melanoma cells were counted under a light microscope at x100 magnification in all fields with triplicate membranes. Values shown represent the mean±SD from triplicate membranes. Increased invasiveness was observed in RNAi-transfected CRL1579 cells compared to control cells ($p=0.03$). However, invasiveness was decreased in SK-MEL-28 ($p<0.001$) and HMV-II cells ($p=0.04$). B. RT-PCR of Microphthalmia-associated transcriptional factor (MITF) expression following HP1β-targeted RNAi transfection in CRL1579, SK-MEL-28, and HMV-II melanoma cell lines. Decreased MITF expression was observed following HP1β-targeted RNAi transfection at the mRNA level in SK-MEL-28 cells compared with control. Nevertheless, in the CRL1579 and HMV-II melanoma cell lines, MITF mRNA expression was up-regulated following HP1β-targeted RNAi transfection.*

Recently, in melanoma models, RNAi-induced changes in *B-RAF* expression have been shown to lead to reversible tumor progression (39). In addition, 57% of melanomas and 100% of melanoma cell lines have been reported to have *RAS*

or *B-RAF* mutations (40). Thus, *B-RAF* mutations are considered to be oncogenic in melanoma. However, *B-RAF* mutations have also been observed in melanocytic naevi: *B-RAF* mutations have been identified in 94% of congenital melanocytic naevi and 28% of dysplastic melanocytic naevi (41). Furthermore, *B-RAF* mutations do not appear to produce ERK activation in melanocytic naevi (42). Therefore, together these reports suggest that *B-RAF* mutations alone may not be sufficient to initiate melanoma.

Mutations in *RAS* or *B-RAF* are also known to influence DNA methylation-induced transcriptional repression *via* chromatin remodeling. In colorectal cancer, *B-RAF* mutations are correlated with high-level promoter methylation of multiple loci (43). Par-4 expression has also been shown to be reduced through methylation at promoter site in *RAS*-transformed cells (44). Finally, it is thought that BRAF-HDAC complexes are associated with gene repression through chromatin remodeling (45, 46). In this study, HP1β expression was decreased in ^{V600E}*B-RAF*-transfected melan-a cells compared to ^{WT}*B-RAF*- or oncogenic *RAS*-transfected melan-a cells. This result suggests that the ^{V600E}*B-RAF* mutation might be initiated prior to HP1β down-regulation and, thus, play a key role during melanoma progression. This hypothesis is consistent with a recent report that *B-RAF* mutations probably arise prior to the development of metastatic lesions in melanoma (47).

The ^{V600E}*B-RAF* mutation stimulates constitutive ERK activity, while B-RAF is not required for ERK signaling in RAS-transformed melanocytes or oncogenic *RAS*-expressing melanoma cells (25). In our study, HP1β expression levels were higher in RAS-expressing cells than in *B-RAF*-transfected cells. This suggests that the distinction between HP1β expression levels in ^{V600E}*B-RAF* mutation- and oncogenic *RAS*-transfected cell lines might reflect different pathways through which constitutive ERK activity is stimulated.

In summary, we showed that HP1β is down-regulated in highly invasive melanoma cell lines. In addition, decreased HP1β expression occurs in ^{V600E}*B-RAF* transfected melan-a cells. Altogether, our results suggest that HP1β may be a useful prognostic indicator for cutaneous melanoma.

Acknowledgements

We thank Drs. Richard Marais and Robert Hayward (Signal Transduction Team, The Institute of Cancer Research, London, UK) for providing the ^{WT}B-RAF (B2), ^{VE}B-RAF (VE16) and ^{G12V}RAS (LTRras) cell lines.

References

1 Brown KE, Baxter J, Graf D, Merkenschlager M and Fisher AG: Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol Cell *3*: 207-217, 1999.

2 Francastel C, Walters MC, Groudine M and Martin DI: A functional enhancer suppresses silencing of a transgene and prevents its localization close to centrometric heterochromatin. Cell *99*: 259-269, 1999.

3 Henikoff S: Position-effect variegation after 60 years. Trends Genet *6*: 422-426, 1990.

4 Ayyanathan K, Lechner MS, Bell P, Maul GG, Schultz DC, Yamada Y, Tanaka K, Torigoe K and Rauscher FJ 3rd: Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. Genes Dev *17*: 1855-1869, 2003.

5 Eissenberg JC, James TC, Foster-Hartnett DM, Hartnett T, Ngan V and Elgin SC: Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. Proc Natl Acad Sci USA *87*: 9923-9927, 1990.

6 Eissenberg JC, Morris GD, Reuter G and Hartnett T: The heterochromatin-associated protein HP-1 is an essential protein in *Drosophila* with dosage-dependent effects on position-effect variegation. Genetics *131*: 345-352, 1992.

7 Lechner MS, Begg GE, Speicher DW and Rauscher FJ 3rd: Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol Cell Biol *20*: 6449-6465, 2000.

8 Ryan RF, Schultz DC, Ayyanathan K, Singh PB, Friedman JR, Fredericks WJ and Rauscher FJ 3rd: KAP-1 corepressor protein interacts and co-localizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. Mol Cell Biol *19*: 4366-4378, 1999.

9 Li Y, Kirschmann DA and Wallrath LL: Does heterochromatin protein 1 always follow code? Proc Natl Acad Sci USA *99*: 16462-16469, 2002.

10 Maison C and Almouzni G: HP1 and the dynamics of heterochromatin maintenance. Nat Rev Mol Cell Biol *5*: 296-304, 2004.

11 Muchardt C, Guilleme M, Seeler JS, Trouche D, Dejean A and Yaniv M: Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. EMBO Rep *3*: 975-981, 2002.

12 Meehan RR, Kao CF and Pennings S: HP1 binding to native chromatin *in vitro* is determined by the hinge region and not by the chromodomain. EMBO J *22*: 3164-3174, 2003.

13 Sugimoto K, Yamada T, Muro Y and Himeno M: Human homolog of *Drosophila* heterochromatin-associated protein 1 (HP1) is a DNA-binding protein which possesses a DNA-binding motif with weak similarity to that of human centromere protein C (CENP-C). J Biochem (Tokyo) *120*: 153-159, 1996.

14 Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC and Kouzarides T: Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*: 120-124, 2001.

15 Lachner M, O'Carroll D, Rea S, Mechtler K and Jenuwein T: Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*: 116-120, 2001.

16 Nakayama J, Rice JC, Strahl BD, Allis CD and Grewal SI: Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*: 110-113, 2001.

17 Singh PB, Miller JR, Pearce J, Kothary R, Burton RD, Paro R, James TC and Gaunt SJ: A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. Nucleic Acids Res *19*: 789-794, 1991.

18 Minc E, Courvalin JC and Buendia B: HP1gamma associates with euchromatin and heterochromatin in mammalian nuclei and chromosomes. Cytogenet Cell Genet *90*: 279-284, 2000.

19 Nielsen AL, Oulad-Abdelghani M, Ortiz JA, Remboutsika E, Chambon P and Losson R: Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. Mol Cell *7*: 729-739, 2001.

20 Le Douarin B, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R and Chambon P: A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. EMBO J *15*: 6701-6715, 1996.

21 Verschure PJ, van der Kraan I, de Leeuw W, van der Vlag J, Carpenter AE, Belmont AS and van Driel R: *In vivo* HP1 targeting causes large-scale chromatin condensation and enhanced histone lysine methylation. Mol Cell Biol *25*: 4552-4564, 2005.

22 Kirschmann DA, Lininger RA, Gardner LM, Seftor EA, Odero VA, Ainsztein AM, Earnshaw WC, Wallrath LL and Hendrix MJ: Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer. Cancer Res *60*: 3359-3363, 2000.

23 van Elsas A, Zerp SF, van der Flier S, Kruse KM, Aarnoudse C, Hayward NK, Ruiter DJ and Schrier PI: Relevance of ultraviolet-induced *N-ras* oncogene point mutations in development of primary human cutaneous melanoma. Am J Pathol *149*: 883-893, 1996.

24 Tsao H, Zhang X, Fowlkes K and Haluska FG: Relative reciprocity of *NRAS* and *PTEN/MMAC1* alterations in cutaneous melanoma cell lines. Cancer Res *60*: 1800-1804, 2000.

25 Wellbrock C, Ogilvie L, Hedley D, Karasarides M, Martin J, Niculescu-Duvaz D, Springer CJ and Marais R: *V599EB-RAF* is an oncogene in melanocytes. Cancer Res *64*: 2338-2342, 2004.

26 Wellbrock C and Marais R: Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. J Cell Biol *170*: 703-708, 2005.

27 Widlund HR and Fisher DE: Microphthalamia-associated transcription factor: a critical regulator of pigment cell development and survival. Oncogene *22*: 3035-3041, 2003.

28 Salti GI, Manougian T, Farolan M, Shilkaitis A, Majumdar D and Das Gupta TK: Micropthalmia transcription factor: a new prognostic marker in intermediate-thickness cutaneous malignant melanoma. Cancer Res *60*: 5012-5016, 2000.

29 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W *et al*: Mutations of the *BRAF* gene in human cancer. Nature *417*: 949-954, 2002.

30 Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, Moses TY, Hostetter G, Wagner U, Kakareka J *et al*: High frequency of *BRAF* mutations in naevi. Nat Genet *33*: 19-20, 2003.

31 Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, Van Belle P, Elder DE and Herlyn M: Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both *BRAF* mutations and autocrine growth factor stimulation. Cancer Res *63*: 756-759, 2003.

32 Dong J, Phelps RG, Qiao R, Yao S, Benard O, Ronai Z and Aaronson SA: BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. Cancer Res *63*: 3883-3885, 2003.

33 Bradbury J: RNA interference: new drugs on the horizon. Drug Discov Today *10*: 1266-1267, 2005.

34 Hammond SM: Dicing and slicing – the core machinery of the RNA interference pathway. FEBS Lett *579*: 5822-5829, 2005.

35 Lu PY, Xie F and Woodle MC: *In vivo* application of RNA interference: from functional genomics to therapeutics. Adv Genet *54*: 117-142, 2005.

36 Sandy P, Ventura A and Jacks T: Mammalian RNAi: a practical guide. Biotechniques *39*: 215-224, 2005.

37 Hwang KK and Worman HJ: Gene regulation by human orthologs of *Drosophila* heterochromatin protein 1. Biochem Biophys Res Commun *293*: 1217-1222, 2002.

38 Sharma GG, Hwang KK, Pandita RK, Gupta A, Dhar S, Parenteau J, Agarwal M, Worman HJ, Wellinger RJ and Pandita TK: Human heterochromatin protein 1 isoforms HP1(Hsalpha) and HP1(Hsbeta) interfere with hTERT-telomere interactions and correlate with changes in cell growth and response to ionizing radiation. Mol Cell Biol *23*: 8363-8376, 2003.

39 Hoeflich KP, Gray DC, Eby MT, Tien JY, Wong L, Bower J, Gogineni A, Zha J, Cole MJ, Stern HM, Murray LJ, Davis DP and Seshagiri S: Oncogenic *BRAF* is required for tumor growth and maintenance in melanoma models. Cancer Res *66*: 999-1006, 2006.

40 Reifenberger J, Knobbe CB, Sterzinger AA, Blaschke B, Schulte KW Ruzicka T and Reifenberger G: Frequent alterations of *Ras* signaling pathway genes in sporadic malignant melanomas. Int J Cancer *109*: 377-384, 2004.

41 Papp T, Schipper H, Kumar K, Schiffmann D and Zimmermann R: Mutational analysis of the *BRAF* gene in human congenital and dysplastic melanocytic naevi. Melanoma Res *15*: 401-407, 2005.

42 Uribe P, Andrade L and Gonzalez S: Lack of association between *BRAF* mutation and MAPK ERK activation in melanocytic nevi. J Invest Dermatol *126*: 161-166, 2006.

43 Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, Kambara T, MacPhee DG, Young J, Leggett BA, Jass JR, Tanaka N and Matsubara N: Colorectal cancer with mutation in *BRAF*, *KRAS*, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol *222*: 4584-4594, 2004.

44 Pruitt K, Ulku AS, Frantz K, Rojas RJ, Muniz-Medina VM, Rangnekar VM, Der CJ and Shields JM: *Ras*-mediated loss of the pro-apoptotic response protein Par-4 is mediated by DNA hypermethylation through *Raf*-independent and *Raf*-dependent signaling cascades in epithelial cells. J Biol Chem *280*: 23363-23370, 2005.

45 Hakimi MA, Bochar DA, Chenoweth J, Lane WS, Mandel G and Shiekhattar R: A core-BRAF35 complex containing histone deacetylase mediates repression of neuronal-specific genes. Proc Natl Acad Sci USA *99*: 7420-7425, 2002.

46 Iwase S, Januma A, Miyamoto K, Shono N, Honda A, Yanagisawa J and Baba T: Characterization of BHC80 in BRAF-HDAC complex, involved in neuron-specific gene repression. Biochem Biophys Res Commun *322*: 601-608, 2004.

47 Libra M, Malaponte G, Navolanic PM, Gangemi P, Bevelacqua V, Proietti L, Bruni B, Stivala F, Mazzarino MC, Travali S and McCubrey JA: Analysis of *BRAF* mutation in primary and metastatic melanoma. Cell Cycle *4*: 1382-1384, 2005.

Received June 9, 2006
Accepted August 18, 2006