

Epigenetic Silencing of the *PTEN* Gene in Melanoma

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Abstract

Phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*) seems to be an important tumor suppressor gene in melanoma. Because the *PTEN* gene is only infrequently deleted or mutated, and because the *PTEN* protein is low to absent in a significant number of melanomas, we investigated alternative methods of epigenetic silencing. We did quantitative positional methylation analysis (pyrosequencing) on 37 sera from melanoma patients and on 21 pairs of corresponding sera and melanoma specimens in addition to Taqman reverse transcription-PCR. We report significant positional *PTEN* promoter methylation in 62% of circulating DNA isolated from sera of patients with metastatic melanoma. The percentage of methylation of a selected CpG island in blood showed a correlation with methylation levels in the corresponding melanoma tissue. Moreover, high percentages of *PTEN* methylation were associated with low *PTEN* transcription levels. Using the demethylation agent 5-aza-2'-deoxycytidine, reduced methylation and a corresponding increase in *PTEN* protein were observed in BLM melanoma cells, leading to reduced AKT activity in an *in vitro* kinase assay. In summary, epigenetic *PTEN* silencing seems to be a relevant mechanism of inactivating this tumor suppressor gene in melanoma that may promote melanoma development by derepression of the AKT pathway. (Cancer Res 2006; 66(13): 6546-52)

Introduction

Loss of tumor suppressor genes on chromosome 10 plays an important role in the development of 30% to 50% of melanomas. In particular, loss of the *phosphatase and tensin homologue deleted from chromosome 10 (PTEN)* is an important gene, of which the loss has been reported to contribute to the development of noninherited melanomas (1). The *PTEN* gene encodes a phosphatase that degrades the product of phosphatidylinositol 3-kinase (PI3K) by dephosphorylating phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate at the 3' position (2). The loss of function of *PTEN* from tumor cells causes accumulation of these critical messenger lipids, which in turn increase AKT phosphorylation and activity, leading to decreased apoptosis and/or increased mitogen signaling (3-7).

Note: A. Mirmohammadsadegh and A. Marini contributed equally to this study.

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Besides the loss of the *PTEN* gene, several *PTEN* mutations have been identified (8, 9). However, whereas mutations and deletions of *PTEN* have been observed in up to 60% of melanoma cell lines, only about 10% of uncultured tumor material were found to harbor genetic alterations (10-16). These observations have led to speculations that *PTEN* inactivation may predominantly occur through a mechanism other than mutation for example by epigenetic silencing (17). Although epigenetic silencing of the *PTEN* gene has been shown in advanced human prostate cancer (18) and hematologic malignancies (19) as well as endometrial carcinoma (20), it has not been analyzed in malignant melanoma. Interestingly, a recent report has shown altered *PTEN* protein expression in 43% of 27 patients with primary melanoma (21). In an earlier report, Zhou et al. detected absent or low *PTEN* expression in 65% of melanoma metastases despite wild-type *PTEN* (22). These results suggested an epigenetic mechanism of biallelic functional inactivation.

Therefore, we thought to determine whether epigenetic modification of *PTEN* occurred in primary melanoma and melanoma metastases using both quantitative and positional methylation analysis. Besides analyzing tumor specimens, the methylation profile of small circulating DNA promoter fragments in the corresponding serum of the same patients was also investigated. We identified several distinct methylation sites within the *PTEN* promoter, leading to reduced *PTEN* expression in melanoma.

Materials and Methods

Patients and serum samples. A total of 37 peripheral blood specimens from 26 patients with primary melanoma and 11 with melanoma metastases were analyzed. For comparison, sera from nine healthy controls without nevi and nine individuals with >10 nevi of >5 mm in size were used as controls. We also compared patients' sera ($n = 21$) with their corresponding primary melanoma ($n = 13$) and melanoma metastases ($n = 8$) obtained at the time of excision to those of healthy controls (Table 1). In addition, we examined normal human melanocytes (NHM) and several melanoma cell lines (SK-Mel-28, BLM, MV3, and M13). The experiments received institutional ethical approval, and patients consented to the blood analysis. The protocol adhered to the Helsinki Guidelines.

Sample preparation. Genomic DNA was isolated from 1 mL serum using QIAamp Ultrasense blood mini kit (Qiagen, Hilden, Germany) and from 21 formalin-fixed melanoma tissue specimens using the QIAamp DNA Micro kit (Qiagen) according to the manufacturer's protocol. DNA samples were kept frozen at -70°C until use. From these 21 melanoma samples, frozen material was also available and used for RNA analysis.

RNA isolation from tissue and quantitative real-time reverse transcription-PCR. Total RNA was extracted from frozen tumor tissue and control skin using Trizol according to the manufacturer's recommendation (Invitrogen/Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized in 20- μL reaction volume containing 5 mmol/L MgCl_2 , 1 mmol/L deoxynucleotide triphosphate mix, $1 \times$ PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3)], 2.5 mmol/L random hexamer, 1 unit/ μL RNA guard, 2.5 units/ μL MuLV reverse transcriptase, and 1 μg of total RNA.

Table 1. Clinical characteristics

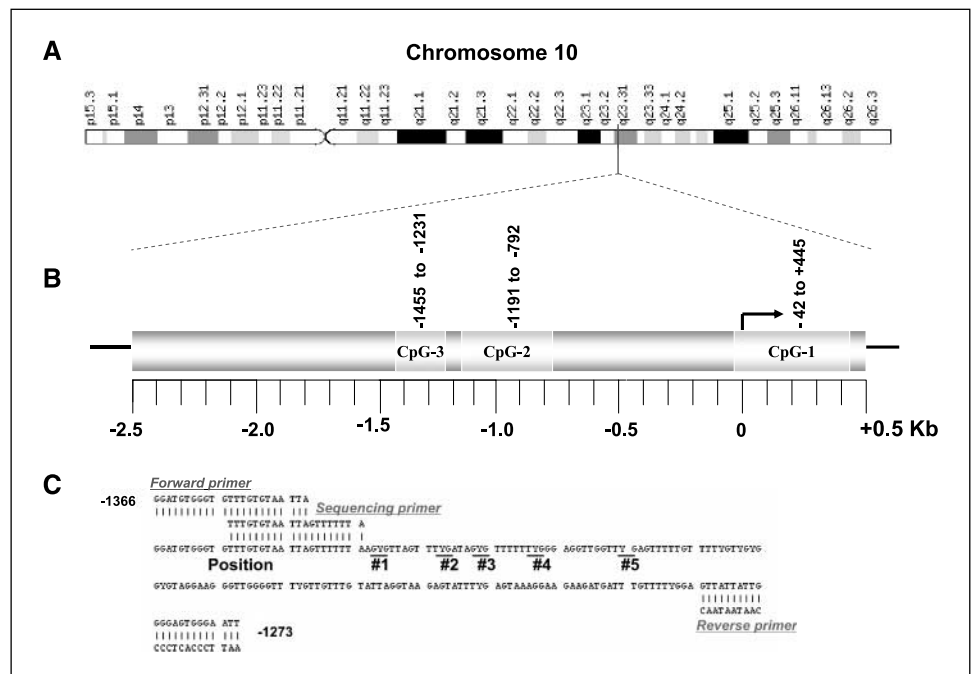
No.	Age	Gender	Diagnosis	Localization of metastases			
1	78	F	Metastatic melanoma	Cutaneous, lymph node and breast			
2	68	F	Metastatic melanoma	Cutaneous			
3	77	F	Metastatic melanoma	Cutaneous			
4	75	F	Metastatic melanoma	Cutaneous, lymph node and breast			
5	75	F	Metastatic melanoma	Cutaneous			
6	81	F	Metastatic melanoma	Cutaneous, lymph node and breast			
7	68	F	Metastatic melanoma	Cutaneous			
8	72	F	Metastatic melanoma	Cutaneous, lymph node			

No.	Age	Gender	Diagnosis	Clark level	Breslow (mm)	Year of first diagnosis	Localization of tumor
9	61	M	Primary melanoma	IV	0.8	09/04	Upper arm
10	65	F	Primary melanoma	III	0.82	09/04	Back
11	71	M	Primary melanoma	IV	4.2	09/04	Back of the left hand
12	78	F	Primary melanoma	IV	3.24	05/03	Lower right leg
13	73	M	Primary melanoma	IV	0.55	06/04	Abdomen right
14	66	F	Primary melanoma	III-IV	0.5	05/04	Scalp
15	67	F	Primary melanoma	IV	4.23	03/05	Back
16	62	M	Primary melanoma	IV	1.6	05/05	Lower right leg
17	40	F	Primary melanoma	IV	4.0	06/05	Scalp
18	70	M	Primary melanoma	III-IV	0.82	07/05	Left arm
19	72	M	Primary melanoma	II-III	0.9	08/05	Scalp
20	66	F	Primary melanoma	II	0.45	08/05	Lower leg
21	69	M	Primary melanoma	IV	2.0	06/04	Lower back

Samples were incubated for 10 minutes at 20°C and for 15 minutes at 42°C followed by 5 minutes at 99°C. Transcript analysis of fresh tumors was done by real-time reverse transcription-PCR using the Taqman assay. Hybridization probes and primer were chosen from the online catalogue of Applied Biosystems (Foster City, CA). Typically, PCR was carried out in a 96-well plate containing 25 µL volumes. A fluorescent dye, 6-carboxyl-X-

rhodamin, was included in the Taqman buffer to serve as an internal reference. Thermal cycling was initiated with a first denaturation step for 10 minutes at 95°C followed by 50 cycles done in two steps: for 15 seconds at 95°C and for 1 minute at 60°C. All samples were amplified simultaneously in duplicates in an one-assay run. Variation coefficients for C_T values for duplicate reactions were excellent (<2% for all gene products).

Figure 1. Detailed map of chromosome 10. A, chromosomal localization of the *PTEN* gene. B, using the Gene2Promoter software and the EMBLEBI-CpG-PLOT, three CpG islands (CpG-1, CpG-2, and CpG-3) were identified and are depicted with regard to the transcription start site. Five CpG sites (sites 1-5) were identified within the CpG-3 island. C, forward, reverse, and sequencing primers used for the detection of CpG positions within the CpG-3 island of the *PTEN* sequence.



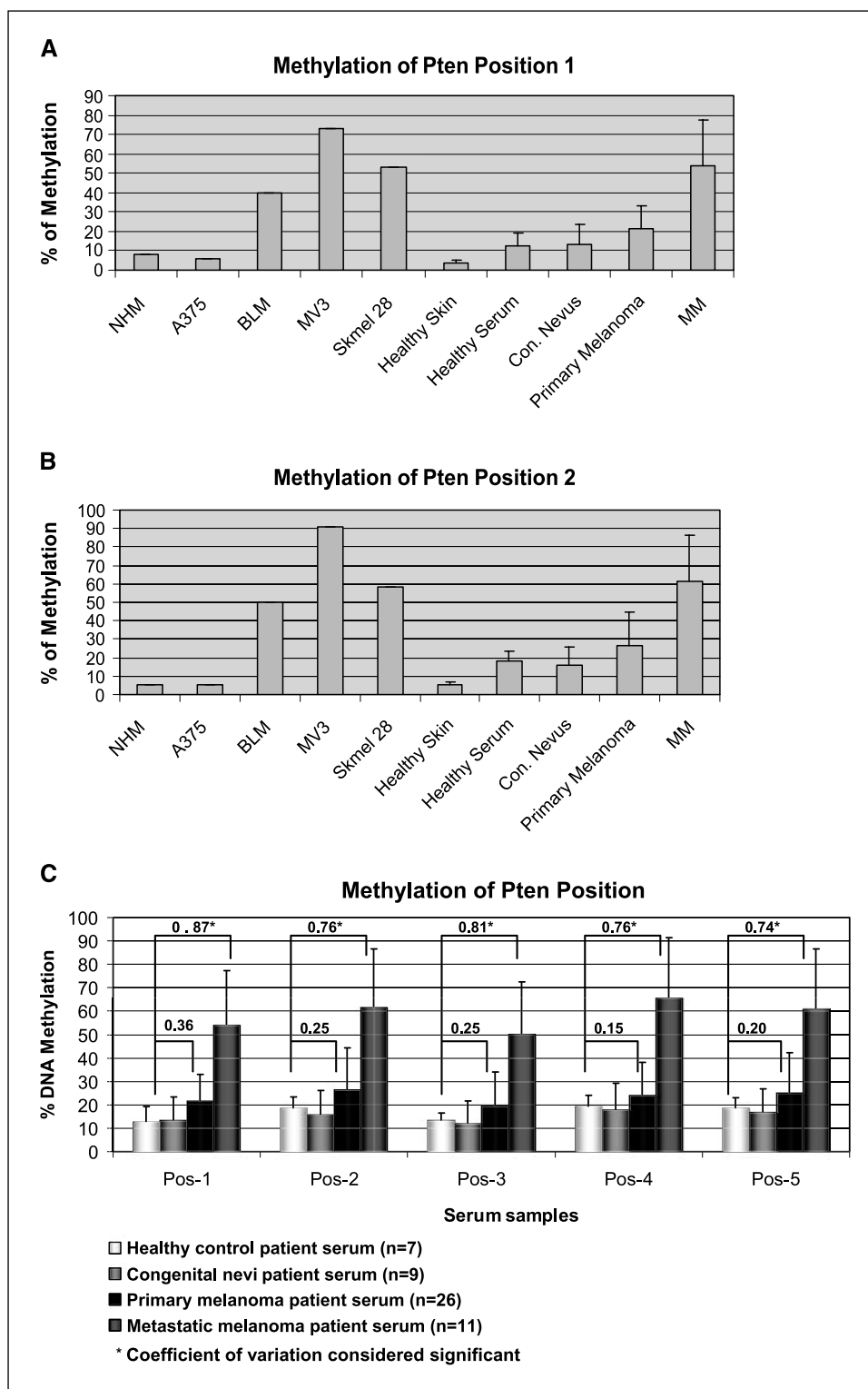


Figure 2. Positional methylation of CpG-3 within the *PTEN* promoter. *A*, methylation frequency of the position 1 of primary melanoma, melanoma metastases, benign nevi, and healthy patients serum. Sera from melanoma metastasis patients were significantly different from those of congenital nevi and healthy serum ($P < 0.05$). *B*, positional methylation of the CpG position 2 in patients' sera as described above. *C*, positional methylation of the CpG positions 1 to 5 of CpG-3 in melanoma patients' sera and several control patients. The pattern of methylation was similar for all five positions (positions 1-5). *, the coefficient of variation is considered significant for metastatic melanoma compared with healthy controls.

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Analysis of DNA methylation. One microgram of DNA isolated from paraffin-fixed tissue was used for bisulfite treatment done by the EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the supplier's protocol.

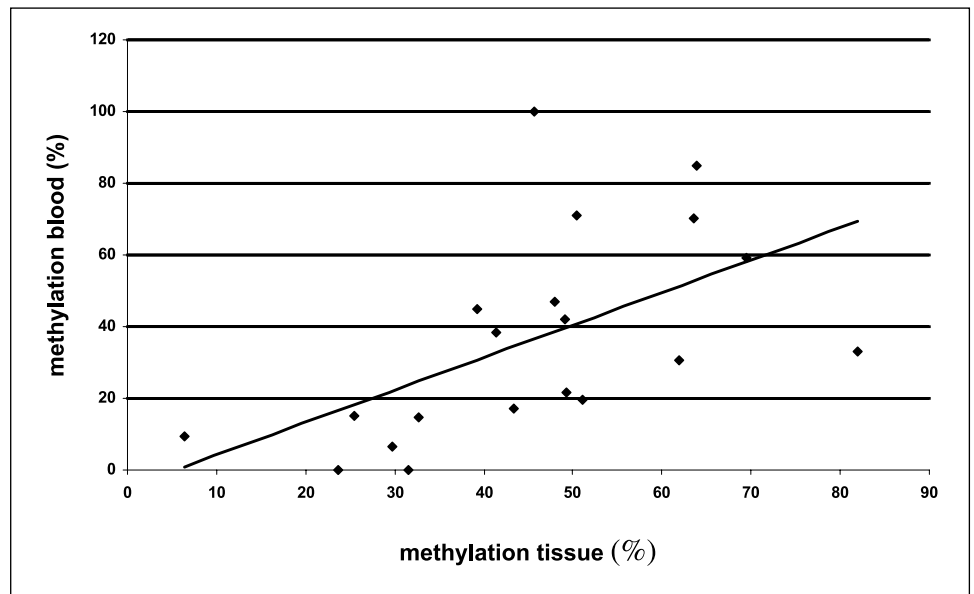
Pyrosequencing. The Gene2Promoter software (Genomatix Software, Munich, Germany)³ allows the automated selection of promoters from a list

of accession numbers or gene IDs. We analyzed potential promoter regions spanning 2,500 bp upstream and 500 bp downstream around the transcription start site of the *PTEN* gene. Subsequently, CpG islands were identified within this core promoter region using the European Molecular Biology Laboratory EBI-CpG plot (Fig. 1).⁴ For primer design, the DNA

³ <http://www.genomatix.de>.

⁴ <http://www.ebi.ac.uk/emboss/cpgplot/index.html>.

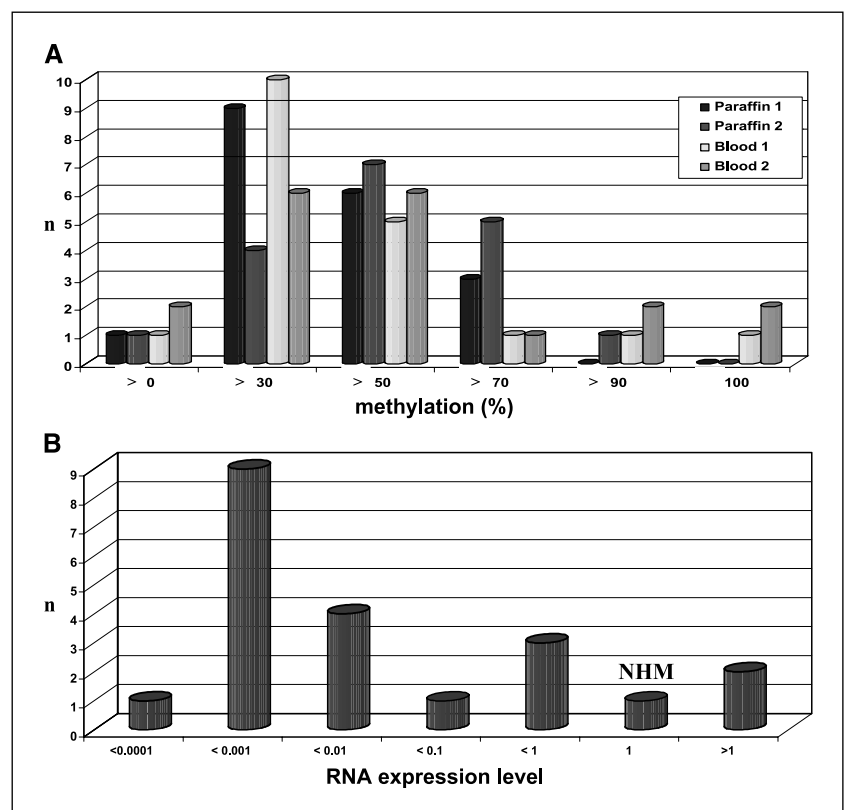
Figure 3. Correlation of methylation in blood and paraffin-embedded tissue for position 2 of CpG-3 of the *PTEN* promoter. The Pearson's correlation coefficient was 0.45.



sequences were converted *in silico* to the methylated form of CpG as follows: CG motifs were converted to YG with Y equaling either C/T or G/A, and subsequently, C was converted to T. Using this converted sequence, methylation-specific primers for quantitative sequencing (pyrosequencing) of *PTEN* CpGs were designed using the Biotage Assay Design software and pyrosequencer PSQ HS96 (Biotage, Uppsala, Sweden) as follows: forward primer, GGATGTGGGTGTTGTGTAATTA; reverse primer, Biotin-AATCCCACTCCCAATAATAAC (reverse complementary); sequencing primer, TTTGTGTAATTAGTTTTTTA; sequence to analyze, AGYGTAGTTTYGATAGYGTTTTTTYGGGAGGTTGGTTYG.

PCR conditions for pyrosequencing. Fifty nanograms of bisulfite-treated DNA were used in the PCR reaction with 200 nmol/L forward and reverse primers, respectively. PCR conditions for *PTEN* were 1 × 95°C for 15 minutes (95°C for 40 seconds, 55°C for 40 seconds, and 72°C for 40 seconds), 50 cycles and 1 × 72°C for 10 minutes using HotStar Taq (Qiagen). For statistical analysis, the % coefficient of variation was calculated for *PTEN* methylation of healthy individuals versus patients with nevi, melanoma, and melanoma metastases, respectively. A % coefficient of variation greater than the threshold of 20 was considered significant.

Figure 4. Correlation of CpG-3 methylation with *PTEN* expression. The rate of methylation for the representative CpG positions 1 and 2 is depicted for paraffin-embedded tissue (*paraffin*) and circulating blood (*blood*). *A*, generally, high rates of methylation in excess of 30% were detected for both positions shown in paraffin and blood. *B*, the RNA expression level in the corresponding frozen melanoma tissue was analyzed by Taqman PCR. The expression of *PTEN* RNA in the analyzed samples was very low (10^{-3} to 10^{-4}) relative to NHM RNA.



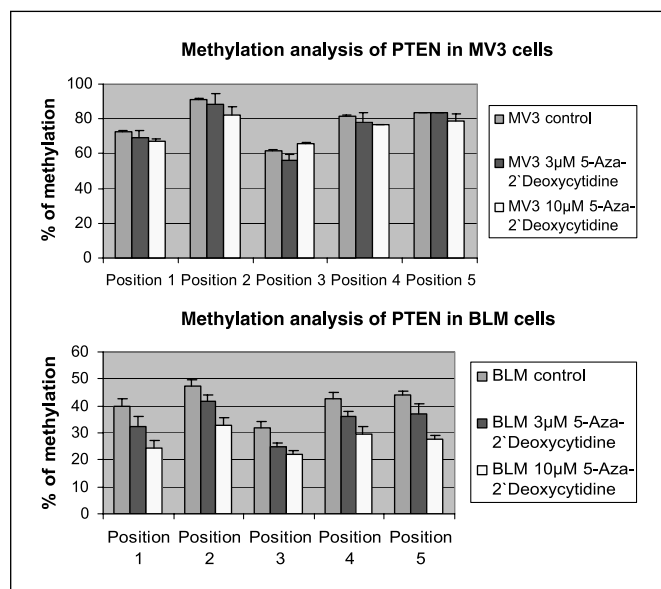


Figure 5. Positional methylation of CpG-3 within the *PTEN* promoter of MV3 cells and BLM cells following demethylation using 5-Aza. In particular, methylation decreased significantly at all sites (sites 1-5) following treatment with 5-Aza (3 and 10 $\mu\text{mol/L}$, 4 days), whereas no change was seen in MV3 cells.

5-Aza-2'-deoxycytidine treatment. Melanoma cell lines MV3 and BLM were incubated with 3 or 10 $\mu\text{mol/L}$ 5-aza-2'-deoxycytidine (5-Aza; Sigma, Taufkirchen, Germany) for 4 days in DMEM with 10% FCS. The medium containing 5-Aza was changed every day.

Western blot analysis. Cells were lysed in buffer containing 25 mmol/L HEPES (pH 7.9), 50 mmol/L NaF, 1% Triton X-100, 5 mmol/L EDTA, 100 mmol/L NaCl, and one tablet protease inhibitor cocktail per 10 mL buffer (Roche, Mannheim, Germany). Immunostaining was done using a *PTEN* polyclonal rabbit antibody (Cell Signaling, New England Biolabs, Frankfurt, Germany), AKT antibody (Sc-8312; Santa Cruz Biotechnology, Santa Cruz, CA) and an α -tubulin monoclonal mouse antibody (Oncogene, San Diego, CA), respectively.

In vitro kinase assay. AKT *in vitro* kinase assay was done using protein lysates prepared from MV3 and BLM melanoma cell lines before and after the treatment with the indicated concentration of 5-Aza as described (23). Briefly, cell cultures were washed in ice-cold PBS and lysed in ice cold buffer L [20 mmol/L HEPES (pH 7.9), 10 mmol/L EGTA, 40 mmol/L β -glycerophosphate, 25 mmol/L MgCl_2 , 2 mmol/L Na_3VO_4 , 1 mmol/L DTT, 100 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride (pH 8), 1% NP40 and mixed with one protease inhibitor tablet (Roche)]. Protein extracts were clarified by centrifugation (14,000 $\times g$, 15 minutes, 4°C). Cell lysates (~100 μg) were incubated with anti-AKT antibody (Sc-8312; Santa Cruz Biotechnology) for 1 hour at 4°C. The immune complexes were bound to Sepharose protein A (5 mg/mL in lysis buffer) by rotating overnight at 4°C. After centrifugation, the Sepharose beads were washed thrice with kinase reaction buffer [80 mmol/L HEPES (pH 7.9), 80 mmol/L MgCl_2 , 0.1 mmol/L ATP, 2 mmol/L Na_3VO_4 , and 20 mmol/L NaF]. The activity of the AKT kinase was determined by incubation with 2 μg of recombinant GSK3 protein (Biomol, Hamburg, Germany) as substrate for AKT and 10 μCi of [γ - ^{32}P]dATP (Hartmann Analytika, Munich, Germany) in 15 μL kinase reaction buffer and a subsequent incubation for 30 minutes at 37°C. Reactions were terminated by addition of 15 μL of sample buffer and analyzed by SDS-PAGE gel. The gel was dried and autoradiographed. The expression of AKT was quantified by Western blotting analysis using the same anti-AKT antibody according to standard procedures.

Results

Given the importance of *PTEN* as a potential tumor suppressor gene, we did quantitative positional methylation analysis

(pyrosequencing) of *PTEN* within and upstream of the promoter region as predicted by appropriate algorithms (Fig. 1). As we and others have recently reported the analysis of genes in the serum for example in patients with breast cancer (24, 25) or melanoma (26), we screened circulating DNA isolated from sera of melanoma patients for *PTEN* methylation at various positions (Fig. 2A and B). Fifty-four percent and 62 % of patients with metastatic melanoma harbored methylated CpG sites 1 and 2 within CpG-3 as opposed to 22% and 26% of patients with primary melanoma, respectively (Fig. 2A and B; $P < 0.05$). Interestingly, similar percentages of positional methylation were observed for all five positions analyzed contained in CpG-3 (Fig. 2C). Methylation of CpG occurred in sera of patients with metastatic melanoma in 50%, 66%, and 61% at sites 3, 4, and 5 in CpG-3, respectively (Fig. 2C). Methylation of these sites was generally lower in sera of patients with primary melanoma and in individuals with >10 benign nevi and healthy controls (Fig. 2C). The percent coefficient of variation was >70% between control and metastases and >20% between control and primary melanoma. For comparison, NHM consistently showed methylation rates of below 10% as did A375 cells that are known to harbor the wild-type *PTEN* gene (Fig. 2A and B). In contrast, the melanoma cell line SK-Mel-28 and MV3 that contain a mutated *PTEN* gene showed high methylation rates of between 58% and 91% (Fig. 2A and B).

Next, we were interested to find out, whether the rate of methylation in serum paralleled the rate of methylation in paraffin-embedded sections. This would potentially enable the post hoc analysis of archived tissue samples and the comparison with follow-up examinations using blood. As shown in Fig. 3, there was a (weak) correlation of the methylation at position 2 between circulating cell-free DNA and formalin-fixed tissue (Pearson's correlation analysis, $R = 0.45$). Methylation levels in blood were generally lower than those in tissue specimens. This is not unexpected, as many other sources may shed *PTEN* DNA into the bloodstream.

As we provide quantitative data on positional methylation at certain CpG islands of the *PTEN* promoter, we next asked the question whether methylation at a given position correlated with *PTEN* RNA expression in corresponding frozen tissue of the same patients. Importantly, we found a significant negative correlation between the percentage of methylation in circulating DNA and the level of *PTEN* RNA expression (Fig. 4). From this result, we concluded that the positional methylation of the two indicated CpG sites 1 and 2 of CpG-3 was functionally relevant leading to decreased expression of the *PTEN* gene product.

To evaluate the effect of treatment with the demethylation agent 5-Aza with regard to positional methylation of the five

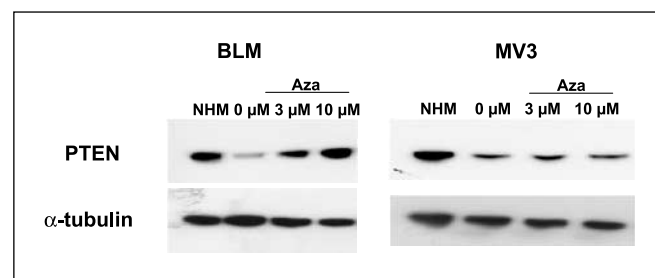
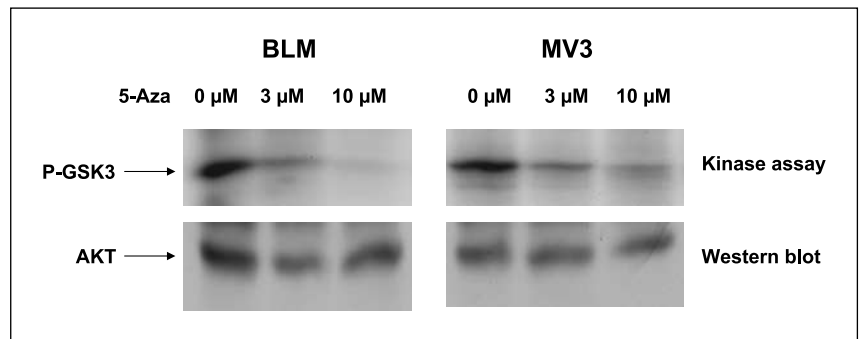


Figure 6. Western blot of *PTEN* protein following treatment of MV3 and BLM cells with 5-Aza (3 and 10 $\mu\text{mol/L}$, 4 days). α -tubulin is shown to confirm equal protein loading.

Figure 7. AKT kinase assay before and following treatment of cells with 5-Aza. The AKT kinase assay showed reduced phosphorylation of its substrate GSK3 upon treatment with 5-Aza (3 and 10 $\mu\text{mol/L}$, 4 days), whereas levels of AKT protein were rather stable.



positions (positions 1-5) within the CpG-3 island, MV3 cells and BLM cells were cultured for 4 days in the presence of 3 and 10 $\mu\text{mol/L}$ 5-Aza. Methylation was found to decrease significantly at all five positions (positions 1-5) in BLM cells, whereas there was no response to this demethylating agent in MV3 cells (Fig. 5). In agreement with increasing demethylation of certain CpG islands within the *PTEN* promoter, the protein concentrations of detectable *PTEN* were found to increase in a dose-dependent manner in BLM cells (Fig. 6).

Finally, we attempted to show a functional link between increasing *PTEN* protein as a consequence of decreasing methylation of CpG islands with the PI3K/AKT pathway. Therefore, an AKT kinase assay before and following treatment of cells with 5-Aza was done (Fig. 7). The AKT kinase assay showed reduced phosphorylation of its substrate GSK3 upon treatment with 5-Aza, whereas levels of AKT protein were rather stable (Fig. 7). These results further strengthen the functional role of *PTEN* demethylation as a negative regulator of AKT.

Discussion

We have shown that positional methylation of CpG islands in the *PTEN* gene leads to reduced *PTEN* expression in melanoma. Therefore, *PTEN* seems to be a relevant candidate tumor suppressor gene in melanoma patients besides *RASSF-1A*, one of the first genes reported to be methylated in 16% to 55% of primary melanomas (27). In addition, the *retinoic acid receptor- β 2* (*RAR- β 2*) and the *O⁶-methylguanine-DNA methyltransferase* (*MGMT*) were reported to be hypermethylated in metastatic melanoma rather than in primary melanoma (28).

Recently, we have reported promoter methylation for different genes, such as the *suppressor of cytokine signaling-1* (*SOCS-1*) and *SOCS-2*, *CDKN2a*, *RASSF1A*, and *MGMT*, in circulating cell-free DNA of patients with primary and metastatic melanoma and found that methylation profiles were distinct and significantly higher among different other skin cancers, such as basal cell cancer and Kaposi sarcoma (26, 28). A very recent article by Mori et al. showed that the detection of circulating methylated DNA in serum can predict the response of stage IV melanoma patients to biochemotherapy and disease outcome (29). In this article, the authors reported that circulating methylated *RASSF1A* was significantly less frequent in responders than in nonresponders. The prognosis of patients with at least one methylated gene in their serum was significantly worse than in patients without methylated genes.

Under physiologic circumstances, the PI3K/*PTEN*/AKT pathway is triggered by paracrine/autocrine activation (e.g., by insulin-like growth factor-I; ref. 30). Multiple connections exist with other

pathways, such as the p53 or target of rapamycin pathways whose signals are integrated by the PI3K pathway (31, 32). Several recent studies have connected two important RAS-signaling pathways (the RAS/RAF/mitogen-activated protein kinase and the RAS/PI3K/*PTEN*/AKT pathways), which are both highly active in primary melanoma (33, 34). The authors showed that melanoma cell lines and melanoma metastases that contained a *PTEN* alteration also harbored an activating *B-RAF* mutation, thereby pointing to a possible cooperation between the B-RAF activation and *PTEN* loss in the development of melanoma (33). In addition, loss of *PTEN* and oncogenic activation of RAS seem to occur in a reciprocal fashion, both of which could cooperate to a loss of *CDKN2a* that contributes to melanoma tumorigenesis (34).

To further study the consequences of epigenetic methylation, we did demethylation using 5-Aza that led to decreased methylation, at least in BLM cells, with a corresponding increase in *PTEN* protein. One functional consequence of increasing *PTEN* expression was reduced AKT activity, whereas AKT protein concentrations remained unaffected. Our results strengthen the functional role of epigenetic *PTEN* inactivation in individuals with wild-type *PTEN* for the strong activation of the AKT pathway leading to down-regulation of the apoptotic pathway in melanoma (1, 15). Although the *PTEN* gene is only rarely mutated in clinical melanoma in contrast to melanoma cell lines (34, 35), the *PTEN* protein expression is lost in 63% of primary melanomas as opposed to only 8% of nevi (35). Therefore, the inactivation of the *PTEN* gene by epigenetic methylation may represent an important feature in the progression of melanocytic nevi to primary melanoma. Serial sampling over extended periods of time will allow to analyze a potential correlation with recurrence-free survival as has been shown for other selected genes in breast cancer patients, where methylation significantly predicted overall survival (24). Future studies will have to look at each *PTEN* mutations, deletions, loss of heterozygosity, and methylation side by side to obtain a clear view on the *PTEN* status for given melanoma patients (36).

In summary, we have identified epigenetic *PTEN* silencing that may contribute to the constitutive activation of the AKT pathway known to promote melanoma development.

Acknowledgments

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