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Differential expression patterns of capping protein, protein phosphatase 1 and casein kinase 1 may serve as diagnostic markers for malignant melanoma

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Abstract

Early and accurate diagnosis of malignant melanoma is critical for patient survival. However, currently used diagnostic markers are insufficiently specific, which limits their utility. We aimed to identify molecular markers that are more specific to malignant melanoma, thereby aiding in melanoma diagnosis and treatment. PCR-based suppression subtractive hybridization was used to identify capping protein Z-line α1 (CAPZA1), protein phosphatase 1 catalytic subunit β isoform (PP1CB), and casein kinase 1 α1 (CSNK1A1) as being differentially expressed between melanoma cells and normal melanocytes. qRT-PCR and Western blot analysis confirmed that these genes were over-expressed in melanoma cells. In addition, immunohistochemical assays revealed that expression of PP1CB and CSNK1A1 was significantly greater in human melanoma specimens than nevi ($p<0.0001$). Combined application of PP1CBand CSNK1A showed high sensitivity and specificity for melanoma. Thus, our data suggest PP1CB and CSNK1A1 are potential biomarkers for distinguishing malignant melanoma from other melanocytic lesions. In addition, because CAPZA1, PP1CB and CSNK1A1 are involved in cell motility, which underlies invasion and metastasis of human cancer, they may also be novel targets for anti-metastatic therapies.

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6. Conflict of interest statement

All authors declare that there are no potential conflicts of interest.

Keywords

melanoma; capping protein; protein phosphatase 1; casein kinase 1

1. Introduction

The incidence of malignant melanoma has been steadily increasing worldwide. In 2009 in the United States, 68,720 people were diagnosed with melanoma and 8,650 died of the disease (1). Although malignant melanoma represents only 5% of all skin cancer diagnoses, it is responsible for more than 50% of the deaths caused by skin cancers (2), underscoring the importance of melanoma diagnosis, staging, and treatment. Early detection and surgical excision of the tumor lead to effective cures (3, 4). However, metastatic melanoma remains generally incurable, is largely resistant to current therapies, has a median survival time of only 6 to 9 months, and a 5-year survival rate of 5.5% (5, 6). Because metastatic melanoma is one of the most aggressive types of cancer, early and accurate diagnosis is vital to patient survival.

The disparity in prognosis between localized, early stage melanoma and metastatic melanoma is substantial (3). Early detection, which allows for complete resection of melanoma skin growths at the T1 stage (thinner than 1 mm), results in near complete cure, whereas inoperable late stage metastatic melanoma is incurable. Because metastatic disease is largely refractory to chemotherapy and adjuvant immunotherapeutic agents, diagnosing melanoma during the window of opportunity to resect the tumor and prevent the spread of tumor cells is critical. In addition, cells become motile during metastatic transformation; therefore, prevention of cell mobility is an important strategy for the development of new therapeutic approaches.

Currently, in clinical practice, the most often used diagnostic markers for malignant melanoma are S100, HMB45, and Melan-A (7). However, these markers are insufficiently specific, which limits their value as diagnostic agents (8). The problems caused by misdiagnosing a nevus as melanoma or *vice versa* can have deleterious consequences for both the patient and the pathologist (9). Therefore, more specific molecular markers should be used in parallel with S100, HMB45, and Melan-A to reliably distinguish melanoma from other malignancies and melanocytic lesions. The goal of this study was to identify molecular markers that are more specific to malignant melanoma, thereby aiding in the diagnosis and treatment of this disease.

Here, we report the identification of proteins that are considerably up- or down-regulated in melanoma, which suggests their potential use as diagnostic and prognostic biomarkers and targets for drug design. By using PCR-based suppression subtractive hybridization, which specifically identifies mRNA molecules that are differentially expressed, we identified three genes, capping protein Z-line α1 (CAPZA1), protein phosphatase 1 catalytic subunit β isoform (PP1CB), and casein kinase 1 α1 (CSNK1A1), that are over-expressed at both the mRNA and protein levels in melanoma cells as compared to normal melanocytes. Furthermore, immunohistochemical analyses using antibodies against PP1CB and CSNK1A1 revealed that human melanoma specimens contained significantly more positively-stained cells that stained more intensely than did benign nevi. However, immunostaining with an anti-CAPZA1 antibody did not show a statistically significant difference between melanoma specimens and benign nevi. Taken together, these findings suggest that PP1CB and CSNK1A1 are strong candidates for further studies as diagnostic markers and, together with CAPZA1, may be potential targets for anti-metastatic therapies

for malignant melanoma, since these genes have roles in the cytoskeletal network and cell mobility (10–12).

2. Materials and Methods

2.1 Cell culture

Three malignant melanoma cell lines, Hs294T, HMCB and G-361, were graciously provided by Dr. Warren Chow (City of Hope). A normal human melanocyte cell line and four melanoma cell lines (A7, A375, C32TG and WM2664) were purchased from American Type Culture Collection (ATCC). Melanocytes were cultured in Dermal Cell Basal Medium (ATCC) supplemented with the Melanocyte Growth Kit (ATCC). Melanoma cell lines were cultured in Dulbecco's modified Eagle's medium (Mediatech, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were grown at 37°C, 5% CO_2.

2.2 cDNA synthesis from normal melanocytes and melanoma cell lines

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Poly-A RNA was purified from total RNA with the Oligotex mRNA Mini Kit (Qiagen). To create single-stranded cDNA, Poly-A RNA was reverse transcribed. Briefly, reactions were comprised of Poly-A RNA (2 μg), first strand buffer, 1 mM DTT, 1 mM dNTP mix and 1 mM cDNA synthesis primer (SSH kit, Clontech). For each reaction, 20 units of AMV reverse transcriptase (SSH kit, Clontech) were added and the reverse transcription reaction was carried out for 2 h at 42°C. To synthesize the second-strand DNA, the first single-stranded cDNA was incubated with 1 mM dNTP mix, 1 mM DTT, RNAse H (1 unit), DNA polymerase I (24 units) and *E. coli* ligase T4 (4.8 units). Reactions were brought up to 100 μl with distilled water (30 μl) and incubated (16°C, 2 h). After addition of T4 DNA polymerase (6 units), mixtures were incubated at 16°C for an additional 30 min.

2.3 PCR-based suppression subtractive hybridization

Total cDNA from normal melanocytes and melanoma cell lines was digested (2 h, 37°C) with *RsaI* (New England Biolabs Inc.). After *RsaI*-digestion, the tester cDNA (melanocyte or melanoma cDNA) fragments were divided into two aliquots. One aliquot was ligated with adapter 1, and the other with adapter 2R (SSH kit, Clontech). Two-step hybridization was then performed. In the first hybridization, an excess amount of driver (melanocyte or melanoma cDNA) was added to each tester, and the samples were denatured (1.5 min, 98°C) and then annealed (8 h, 68°C). During the second hybridization, the two primary hybridized samples were mixed without denaturing. Freshly denatured driver was then added, and the mixtures were hybridized (16 h, 68 °C) (13). Thereafter, the differentially expressed tester sequences were amplified with 2-step PCR (PCR and nested PCR) using the 50× Advantage 2 PCR Polymerase Kit (Clontech).

2.4 Differential expression screening

EagI-digested PCR products were inserted into an *EagI*-site of pBluescript II KS+, and then transformed into DH5α cells. A total of 96 white colonies containing the insertion were selected from each subtraction transformation and sub-cultured (4 h, 37°C) in 96-well microtiter plates containing 100 μl LB/Amp (50 μg/ml) media. The inserts were amplified by colony PCR with T3 and T7 primers using the DH5α cell cultures as templates. Tester-driver inserts and driver-tester inserts were spotted onto duplicate 7× 11cm nylon membranes using a 96-well replicating pin tool and a template was used to array up to 864 individual spots per membrane (V&P Scientific). To identify differential expression between tester and driver, the membranes were hybridized with probes made of unsubtracted tester or

driver cDNA fragments (14–16). A typical hybridization of HMCB/melanocyte is shown in Figure 1.

2.5 Sequence analysis

Differentially expressed inserts were sequenced at the City of Hope DNA Sequencing Facility. BLAST searches were then performed with the insert sequences against the National Center for Biotechnology Information nucleotide database. Fragments that showed high homology with previously described sequences were considered to represent known genes.

2.6 qRT-PCR analysis

To confirm the mRNA expression levels of over-expressed genes identified with subtractive hybridization, qRT-PCR was performed. Total RNA was isolated from melanocytes and melanoma cell lines (RNeasy kit, Qiagen). TheExpress One-Step SuperScript qRT-PCR kit (Invitrogen)was used to reverse-transcribe and amplify 25 ng total RNA per reaction, according to the manufacturer's protocol. ProbeFinder software (Roche Applied Science) was used to design primer sets for CAPZA1, PPP1CB, and CSNK1A1 and to select the respective probes from the Universal Probe Library (Roche). The primer/probe set for β-2 microglobin (B2M) was purchased from Roche. All samples were run in triplicate. Amplifications were performed on a Bio-Rad iCycler iQ5 Multiple-Color Real-time PCR Detection System (Hercules). Data were normalized to B2M expression and calculated with the $2^{-\Delta\Delta Ct}$ method. Gene expression differences between melanoma cells and melanocytes were analyzed by Student's t-test.

2.7 Western blot analysis

Whole cell extracts were prepared with ProteoJET Mammalian Cell Lysis reagent (Fermentas), following the manufacturer's instructions. Total protein concentration was measured using the Bradford method (Bio-Tek Instruments Inc.). Whole cell extracts (20 μg for PP1CB and CSNK1A1, 30 μg for CAPZA1) were separated by 4–15% SDS-PAGE (BioRad) and subsequently transferred onto nitrocellulose membrane (Thermo Scientific). The membrane was then blocked in 5% nonfat milk (room temperature, 1 h), and sequentially incubated (overnight, 4°C) with primary antibodies (rabbit anti-human CAPZA1 [ProteinTech Group], 1:100; rabbit anti-human PP1CB [ProteinTech Group], 1:2000; rabbit anti-human CSNK1A1 [Abnova], 1:100). After 3–5 washes with PBST, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase (anti-rabbit, 1:10000, or anti-mouse, 1:10000; Santa Cruz) for 1 h. After 3–5 washes with PBST, the protein bands were visualized with the enhanced chemiluminescence detection kit (Thermo Scientific). Then, membranes were stripped using Restore PLUS Western Blot Stripping Buffer (Thermo Scientific), and a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:4000, Ambion) was used as the primary antibody for loading control.

2.8 Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks for 39 human melanoma samples were retrospectively retrieved from the Pathology archives of Shengjing Hospital of China Medical University. Twenty additional formalin-fixed paraffin-embedded human melanoma samples and 26 formalin-fixed paraffin-embedded human nevi samples were provided by the Pathology archives of the College of Medicine of University of Arizona, Tucson. Sections (5 μm thick) were stained with the following primary antibodies: rabbit anti-human CAPZA1 polyclonal (3.0 μg/ml, ProteinTech Group); rabbit anti-human PP1CB polyclonal (4.0 μg/ml, ProteinTech Group); and rabbit anti-human CSNK1A1 polyclonal (2.0 μg/ml,

Abnova), and also stained with a house-keeping protein, GAPDH (1.0 μg/ml, ProteinTech Group). Briefly, tissue sections were deparaffinized in xylene and rehydrated in an alcohol gradient. Samples were then quenched in 3% hydrogen peroxide and pretreated (20 min) with steam in DIVA buffer (Biocare) to promote antigen retrieval. After antigen retrieval, slides were incubated (5 min) in Protein Block (DakoCytomation). Then slides were incubated (room temperature, 30 min) with primary antibody, followed by incubation (30 min) with anti-rabbit polymer secondary (DakoCytomation). After washing for 5 min in Dako buffer, slides were incubated with the chromogen diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, and sealed with permanent mounting medium.

2.9 Statistical analysis

Statistical analyses included independent-sample Student's t-test, Chi-square test and Fisher's Exact Test (SPSS 13.0 software). In all assays, $P < 0.05$ was considered statistically significant.

3. Results

3.1 Identification of differentially expressed genes

To identify genes that are differentially expressed in melanoma and melanocytes, two-way subtractive hybridizations were carried out among three human malignant melanoma cell lines (Hs294T, HMCB and G-361) and normal human melanocytes using the PCR-Select™ cDNA Subtraction Kit (Clontech). For melanocyte/Hs294T subtraction, ten genes were found to be over-expressed in the melanoma cell line. Nine genes identified through melanocyte/HMCB subtraction and fourteen genes identified through melanocyte/G361 subtraction were over-expressed in the melanoma cell lines (Table 1). In addition, ten genes were down-regulated in Hs294T, as were five genes in HMCB and fifteen genes in G-361 (Data not shown).

CAPZA1, PP1CB, and CSNK1A1 were over-expressed in all three melanoma cell lines, and were chosen for future investigation. In addition, the melanocyte-specific protein Pmel17 was down-regulated in the three melanoma cell lines, which differed from previous reports that Pmel17 was expressed in other melanoma cell lines (human melanoma cell lines 1123 and M14) (17, 18). Pmel17, like many other proteins, might be expressed distinctly in different melanoma cancer cells, and therefore, was excluded from further study.

3.2 mRNA and protein expression of CAPZA1, PP1CB and CSNK1A1 in melanoma cell lines

To confirm that CAPZA1, PP1CB and CSNK1A1 were over-expressed in melanoma cell lines, we examined mRNA and protein expression by RT-PCR and Western blot, respectively, in the melanoma cell lines Hs294T, HMCB, G-361, A7, A375, C32TG, and WM2664. mRNA expression levels of CAPZA1 and PP1CB were significantly higher ($p<0.05$) in all seven melanoma cell lines as compared with normal melanocytes. CSNK1A1 mRNA levels were higher in six of the melanoma cell lines than in normal melanocytes ($p<0.05$), but not in WM2664 cells ($p=0.079$) (Figure 2A). Furthermore, Western blot analysis showed that CAPZA1, PP1CB and CSNK1A1 were present at higher levels in all melanoma cell lines analyzed as compared with normal melanocytes (Figure 2B).

3.3 Evaluating the diagnostic values of CAPZA1, PP1CB and CSNK1A1 in human malignant melanoma tissues

To preliminarily evaluate the diagnostic efficacy of the above genes, an immunohistochemical assay was optimized for determining the expression of CAPZA1,

PP1CB, and CSNK1A1 in human malignant melanoma samples and benign nevi. Formalin-fixed paraffin-embedded malignant melanoma and benign intradermal nevus tissues were stained with antibodies against CAPZA1, PP1CB, and CSNK1A1 (Figure 3), and cytoplasmic staining for all three proteins was observed in both melanoma and nevus cells. As a control, we also performed immunohistochemical staining for the house-keeping gene, GAPDH, which was expressed equally in nevi and melanoma and at very similar levels among the tissue samples from China and Arizona. Immunostaining was semi-quantitatively evaluated by two independent pathologists (F.L. and Q.H.) in a double blind manner. Target gene expression was scored based on both the percentage of positively stained cells (0, <5% of the cells; 1, 5–10% of the cells; 2, 11–50% of the cells; 3, 51–80% of the cells; 4, >80% of the cells) and the intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong). Finally, a composite score was obtained by multiplying the values of the mean staining intensity and the percentage of positive cells (composite score classifications: negative, 0; weakly positive, 1–4; moderately positive, 5–8; strongly positive, 9–12).

In melanomas, the immunostaining composite score of CAPZA1 was generally moderately positive (20/59 samples had a composite score of 5–8); PP1CB was strongly positive, showing diffuse and homogenous reactivity (35/59 samples had a composite score of 9–12); and CSNK1A1 was generally weakly positive (28/59 samples had a composite score of 1–4) (Table 2). Compared with benign nevi, malignant melanomas had higher expression levels of PP1CB and CSNK1A1 ($p<0.0001$, respectively), as revealed by immunohistochemistry. However, no significant difference in the immunoreactivity composite score ($p=0.21$) was noted for CAPZA1 expression between malignant melanomas and nevi. The threshold value for a positive call was considered as the cut-off point, and to maximize the diagnostic values, optimal cut-off points were selected according to the largest area under the receiver operating characteristic curves. Under optimal cut-off, the sensitivity and specificity of PP1CB were 93.2% and 65.4%, respectively, while those of CSNK1A1 were 88.1% and 88.5%, respectively. These data suggested that PP1CB and CSNK1A1 might be potential biomarkers for distinguishing melanoma from nevi in parallel with currently used biomarkers. In general, the combined application of biomarkers might improve the diagnostic efficiency. If considering a positive result for either PP1CB or CSNK1A1 (combination A) as positive indication of melanoma, the sensitivity and specificity of this combination would be 100% and 57.7%, respectively. However, if a positive result were required for both PP1CB and CSNK1A1 (combination B) in order for the test to be considered positive for melanoma, the sensitivity and specificity would be 64.4% and 96.2%, respectively (Table 3).

4. Discussion

Currently, the most commonly used diagnostic markers for malignant melanoma are S100, HMB45, and Melan-A (7). However, these markers are mainly used for confirming melanocytic lesions, and cannot distinguish between nevi and primary melanomas because they are similarly expressed in both tissue types (19). The misdiagnosis of melanoma is detrimental to both the patient and the pathologist (9), and is the second most common cause of cancer malpractice claims in the United States, next only to breast cancer (20). S100, the most widely used marker for melanoma in the clinic, remains the most sensitive (97–100% sensitivity), but its specificity is limited (75–87%) [7]. Therefore, more specific markers are needed, and should be used in parallel with S100 to reliably distinguish melanoma from other S100-positive malignancies or benign lesions such as nevi.

Here, we have identified three genes (CAPZA1, PP1CB, and CSNK1A1) that are differentially expressed in malignant melanoma cell lines as compared with human normal melanocytes. We demonstrate that these genes are over-expressed in all tested melanoma

cell lines. Furthermore, immunohistochemical staining showed that PP1CB and CSNK1A1 are over-expressed in melanoma tissues as compared with nevi. The melanoma samples included 15 (25%) for stage I melanoma, 16 (27%) for stage II melanoma, 13 (22%) for stage III melanoma and 15 (25) for stage IV melanoma. Careful examination of all the available clinical and protein expression data, revealed no significant association between the stage of melanoma with the expression of candidate protein markers in our samples. Although Western analysis suggested CAPZA1 was highly expressed in melanomas, no significant difference in CAPZA1 expression was detected between malignant melanomas and nevi ($p=0.21$) y immunohistochemistry. This discrepancy may be due to the fact that nevi, a type of benign tumor, express CAPZA1 at levels comparable to melanoma, but not normal melanocytes.

Moreover, use of positive staining for either PP1CB or CSNK1A1 as one kind of combined application would have a sensitivity and specificity of 100% and 57.7%, respectively, and could be used for mass screening for melanoma. Conversely, if a positive result for melanoma required positive staining for both PP1CB and CSNK1A1, the sensitivity and specificity would 64.4% and 96.2%, respectively, which would be appropriate for differentiating nevi from melanoma in the clinic. Thus, PP1CB and CSNK1A1 are promising candidate biomarkers for differential diagnosis of malignant melanomas from nevi, and the assay described herein could be used to assist in the histological diagnosis of melanoma, thereby providing important information to pathologists and other clinicians responsible for caring for patients with melanocytic neoplasms that are difficult to classify.

The genes identified in this study (CAPZA1, PP1CB, and CSNK1A1) have been previously demonstrated to play important roles in the cytoskeletal network and cell mobility, which underlies invasion and metastasis of human cancer (21). CAP facilitates cytoskeletal movement by stabilizing actin filaments after they reach an optimum length to create and reinforce lamellipodia. Thus, CAP is essential for creating a stabilized cytoskeletal structure to induce protrusion and cell extension (10, 22, 23). PP1, on the other hand, is targeted to cytoskeletal microtubules and inhibition of PP1 leads to collapse of the cytoskeletal network. Various proteins, such as tau, p85 and neurabin I, are involved in the recruitment of PP1 to microtubules (12, 24, 25), and that various complexes have evolved to trigger microtubule localization of PP1 speak to its importance as a regulator of cell morphology. Finally, CSNK1 can efficiently phosphorylate nm23 (nonmetastatic clone 23), which is responsible for suppressing cell motility and is implicated in the regulation of metastasis in a variety of human cancers (26). CSNK1-mediated phosphorylation of nm23 results in modulation of the nm23 interaction with h-prune. Formation of the h-prune and nm23 protein complex would reduce the amount of free nm23 in the cells, thus enhancing cell motility (11). Further research on the relation of these three genes with metastasis and survival of patients with melanoma is warranted.

Over-expression of these three mobility related genes may play an important role in cancer cell metastasis and could serve as a marker for metastatic tendency and malignancy. In addition to the potential of PP1CB and CSNK1A1 over-expression for use as diagnostic markers, they, together with CAPZA1, may also serve as potential anti-metastatic targets for treating early stage melanoma. The phosphatase activity of PP1 is of primary interest as an anti-metastatic target. The optimal situation would be to develop PP1 inhibitors that hinder the function of the PP1 catalytic domain (PP1c) when complexed to specific protein modifiers. In this case, modifiers would be proteins known to target PP1 to microtubules. Although this would seem to have the potential for systemic toxicity, which has doomed many kinase-inhibiting drugs in clinical trials, many chemical kinase inhibitors are showing great promise in patient treatment (27–30). Another concern would be that previous drugs developed to target the cytoskeleton have met with failure against melanoma because of

their export by multi-drug resistance proteins, such as p-glycoprotein, multidrug resistance protein 1 and glutathione S-transferases (31–33). It remains to be seen if current PP1c inhibitors are transduced by these proteins.

In our studies, we found that expression of CSNK1A1 RNA and protein is significantly greater in all three melanoma cell lines as compared to melanocytes, as well as in the majority of the melanoma tissue specimens analyzed. In contrast to our study, a recent report showed that CSNK1A1 expression decreased with progression from nevi to primary and metastatic melanoma, likely through mechanisms that involve promoter silencing (34). However, the number of specimens used in those studies was not reported. Sinnberg et al. explained that the enhanced invasive growth of nonmetastatic melanoma cells after CSNK1A1 down regulation is mainly mediated by amplified beta-catenin signaling. However, several studies using patient-derived tumor samples have reported that melanoma progression is associated with the loss of nuclear beta-catenin (35). Further studies are needed to clarify these issues.

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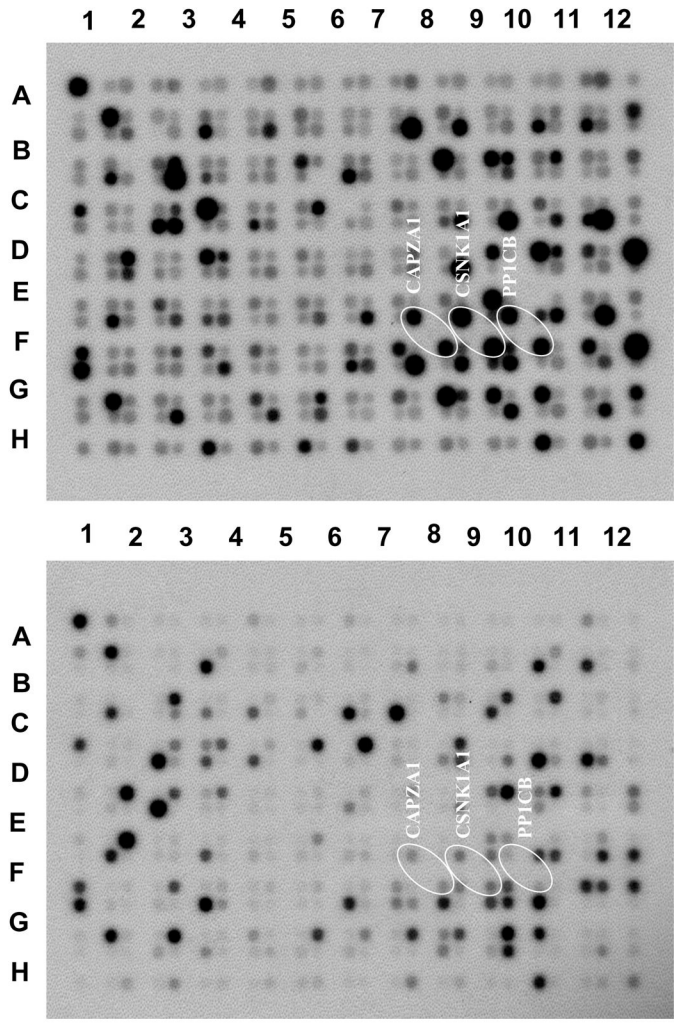

Figure 1. Representative subtractive hybridization of HMCB/melanocyte

The top membrane was hybridized with probes made from unsubtracted tester cDNA fragments. The bottom membrane was hybridized with probes made from driver cDNA fragments. Sequence analysis indicated that clones F8, F9, F10 are CAPZA1, CSNK1A1, and PP1CB, respectively.

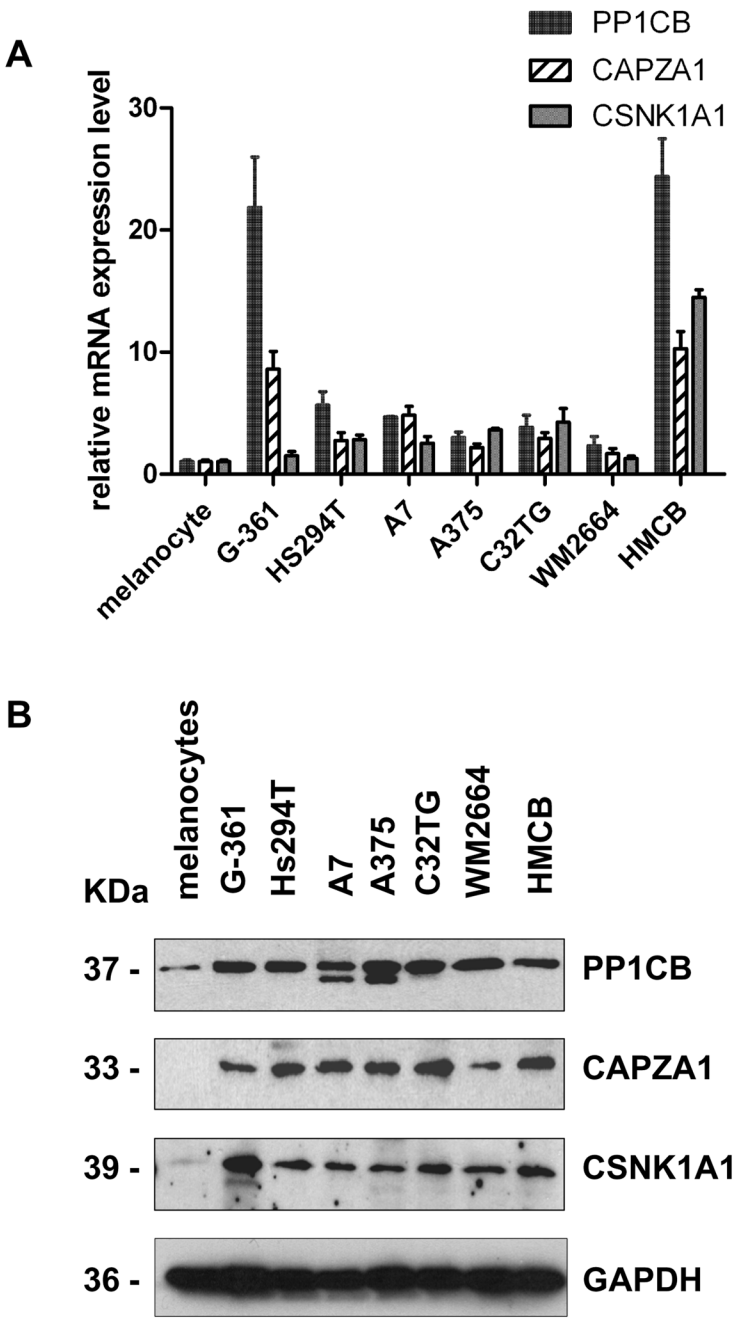

Figure 2. Over-expression of CAPAZ1, PP1CB, and CSNK1A1 mRNA and protein in melanoma cells

(A) qRT-PCR analysis of CAPZA1, PP1CB, and CSNK1A1 mRNA expression in normal melanocytes and seven melanoma cell lines: Hs294T, HMCB, G-361, A7, A375, C32TG and WM2664. CAPZA1 and PP1CB mRNA expression is up-regulated in all seven melanoma lines ($p<0.05$). CSNK1A1 mRNA is up-regulated in six melanoma cell lines ($p<0.05$), except WM2664 ($p=0.079$). (B) Western blot analysis of total protein extracts (20 μg for PP1CB and CSNK1A1; 30 μg for CAPZA1) from the above seven human melanoma cell lines and human melanocytes. The membrane was reblotted with anti-GAPDH a loading

control. The blot shown is representative of at least 3 different independent experiments, all of which gave similar results.

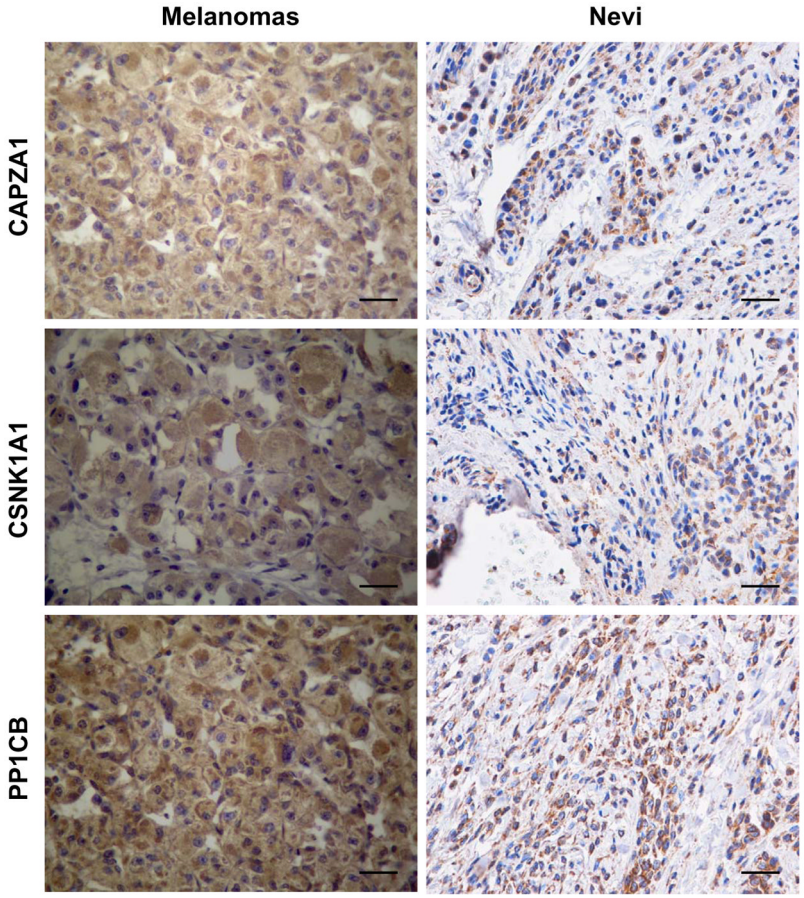

Figure 3. Immunohistochemical analyses of CAPAZ1, PP1CB, and CSNK1A1 in human melanoma tissues and nevi

Representative images of PP1CB expression in melanoma with strong, diffuse staining (200×) (A); PP1CB expression in nevus with weakly positive staining (200×) (B); moderate intensity CAPZA1 staining in melanoma (200×) (C); CAPZA1 expression in nevus with moderately positive staining (200×) (D); CSNK1A1 expression in melanoma with weakly positive staining (200×) (E); and CSNK1A1 expression in nevus with negative staining (200×) (F). For all three proteins, immunostaining was observed in the cytoplasm. Scale bar, 50 μm.

Table 1

Genes identified as over-expressed in three melanoma cell lines as compared with normal melanocytes

Hs294T	HMCB	G-361
Capping protein Z-line alpha 1 (CAPZA1)	Capping protein Z-line alpha 1 (CAPZA1)	Capping protein Z-line alpha 1 (CAPZA1)
Protein phosphatase 1 catalytic subunit beta isoform (PP1CB)	Protein phosphatase 1 catalytic subunit beta isoform (PP1CB)	Protein phosphatase 1 catalytic subunit beta isoform (PP1CB)
Casein kinase 1 alpha 1 (CSNK1A1)	Casein kinase 1 alpha 1 (CSNK1A1)	Casein kinase 1 alpha 1 (CSNK1A1)
FLJ 13207	RP11-320J16 (Chr X)	FLJ 13207
RP11-381P2 (Chr 20)	BAC clone RP11-81F3 (Chr 2)	RP11-401H23 (Chr 9)
FLJ 21487		BAC clone RP11-22N19 from 7q22
Stanniocalcin 1 (STC1)	BAC clone RP11-17P16 (Chr 4)	
KIAA 1581	Nuclear autoantigen (GS2NA)	KIAA 0137
RP5-862K6 (Chr 20)	Vacuolar protein sorting 26	KIAA 0681
RP11-344N17 on (Chr X)	Transcription elongation factor B (TCEB)	RP11-421E17 (Chr 1)
		Radixin
		Blue cone photoreceptor pigment gene
		Protein kinase C binding protein 1 (PRKCBP1)
		Clone CTC-560O9 (Chr 5)
		Clone CTD-2030 (Chr 5)

Table 2

Immunohistochemical staining results for CAPZA1, PP1CB, and CSNK1A1 in human melanomas and nevi

Genes	Composite Score [a]	Melanomas (n=59)	Nevi (n=26)	P Value [b]	Cut-off [c]	Sensitivity (%)	Specificity (%)
CAPZA1							
	0	1	0				
	1–4	9	5		0/1	98.3	0.0
	5–8	20	14		4/5	83.1	19.2
	9–12	29	7	0.211	8/9	49.2	73.1
PP1CB							
	0	0	0				
	1–4	1	3		0/1	100.0	0.0
	5–8	3	14		4/5	98.3	11.5
	9–12	55	9	<0.001	8/9[d]	93.2	65.4
CSNK1A1							
	0	7	23				
	1–4	32	3		0/1[d]	88.1	88.5
	5–8	6	0		4/5	32.2	100.0
	9–12	13	0	<0.001	8/9	22.0	100.0

[a] Composite score was obtained by multiplying the values of the mean staining intensity and the percentage of positive cells (negative, 0; weakly positive, 1 to 4; moderately positive, 5 to 8; strongly positive, 9 to12).

[b] Using Chi-squared and Fisher exact test, $p<0.05$ was considered statistically significant.

[c] Cut-off values represent composite score.

[d] Optimum cut-off points, based on the largest area under receiver operating characteristic curves.

Table 3

Sensitivity and specificity of PP1CB combined with CSNK1A1

Groups	PP1CB	CSNK1A1	Number of cases		Cut-0ff	Sensitivity (%)	Specificity (%)
			Melanoma (n=59)	Nevi (n=26)			
Group 1	−	−	0	15	[a] ←	100	57.7
Group 2	+	−	18	8			
Group 3	−	+	3	2	[b] ←	64.4	96.2
Group 4	+	+	38	1			

[a] Cut-off point between Group 1, in which immunostaining is negative for both proteins, and Groups 2, 3, and 4, in which immunostaining is positive for at least one protein.

[b] Cut-off point between Groups 1, 2, and 3, in which immunostaining for at least one is negative, and Group 4, in which immunostaining for both proteins is positive.