DDEF1 Is Located in an Amplified Region of Chromosome 8q and Is Overexpressed in Uveal Melanoma

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Abstract

Purpose: The molecular pathogenesis of uveal melanoma is poorly understood but is usually accompanied by amplification of chromosome 8q, suggesting the activation of one or more oncogenes. We recently identified a gene expression profile that distinguishes low-grade from high-grade melanomas. In this profile, a cluster of genes at chromosome 8q was overexpressed in high-grade tumors, providing an opportunity to search for potential oncogenes in this region.

Experimental Design: Gene expression microarray analysis was done on 25 primary uveal melanomas. Microarray comparative genomic hybridization (CGH), quantitative PCR, and immunohistochemistry were done on a subset of these tumors. Cell motility was measured using a wound-healing assay.

Results: In melanomas analyzed for microarray gene expression and CGH, gain of chromosome 8q correlated most strongly with expression of DDEF1, a gene located at 8q24. In contrast, the nearby *MYC* oncogene exhibited no significant change in expression. Confirming the microarray findings, DDEF1 mRNA levels and protein expression were significantly higher in high-grade melanomas. Furthermore, ectopic expression of DDEF1 in low-grade melanoma cells resulted in a significant increase in cell motility, a feature of high-grade metastasizing cells.

Conclusions: These findings suggest that DDEF1 overexpression may be a pathogenetically relevant consequence of chromosome 8q amplification, which commonly occurs in high-grade uveal melanomas. We conclude that DDEF1 may act as an oncogene in this cancer, and it may be a useful diagnostic marker and therapeutic target.

Uveal melanoma is the most common ocular cancer and represents about 4% to 5% of all melanomas (1). Nevertheless, the molecular pathogenesis of this neoplasm remains poorly understood. Malignant progression in uveal melanoma is characterized by a transition from low-grade, compact spindle cells to high-grade epithelioid cells that are poorly adherent, highly motile, and prone to metastasize. This tumor progression is usually accompanied by an accumulation of extra copies of chromosome 8q, which is strongly associated with metastatic death (2). The acquisition of additional copies of chromosome 8q during melanoma progression suggests the presence of one or more oncogenes in this region that may be up-regulated during tumor progression. One candidate is the proto-oncogene *MYC*, which is located at 8q24.12-q24.13. In one study using fluorescent *in situ* hybridization, 70% of uveal melanomas exhibited extra copies of the chromosomal region around the *MYC* locus, with frank DNA amplification in 43% of these tumors (3). However, because MYC overexpression does not correlate with melanoma metastasis or tumor-related death (4), the biological relevance of *MYC* in uveal melanoma progression remains uncertain. Further, because the entire 8q chromosomal arm is usually amplified in uveal melanomas (2), it has been difficult to identify other potential oncogenes through the identification of minimally amplified regions.

We recently described a novel molecular classification of uveal melanoma based on gene expression profile that distinguishes low-grade (class 1) from high-grade (class 2) tumors (5). This gene expression profile predicts metastatic death with greater accuracy than other clinical-pathologic factors, and it reveals a cluster of genes on chromosome 8q that is significantly overexpressed in high-grade tumors. These findings corroborate the earlier cytogenetic data and support the notion that one or more oncogenes residing in this region may be overexpressed during melanoma progression.

This microarray gene expression database provided an opportunity for us to search for candidate oncogenes on chromosome 8q in uveal melanoma. Such a discovery could provide a new diagnostic marker, lead to the development of targeted anticancer therapy, and provide new insights into the pathogenesis of this cancer. We show here that *DDEF1*, located at chromosome 8q24, exhibits several features that suggest that it may function as an oncogene during uveal melanoma progression.

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Materials and Methods

Microarray analysis. Gene expression microarray analysis was done on 25 primary uveal melanomas using Affymetrix Hu133A and B GeneChips (Affymetrix, Santa Clara, CA) as previously described (5). Normal uveal melanocytes were obtained from three patients as previously described (6). Institutional Review Board approval was obtained.

Comparative genomic hybridization microarray analysis. These experiments were done as previously described (5). Briefly, genomic DNA was prepared from six uncultured tumor specimens using the DNEasy kit (Qiagen, Valencia, CA). Microarray comparative genomic hybridization (CGH) was done and the log 2 average raw ratio (a measure of DNA copy number with normal 2N = 0) was calculated by the Microarray Shared Resource at the Comprehensive Cancer Center University of California San Francisco, as previously described (7).

Real-time PCR. Quantitative real-time PCR was done on eight tumor samples using the Invitrogen Lux primer system (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer for the Biorad I-cycler (Bio-Rad Laboratories, Hercules, CA). Primers for DDEF1 cDNA were TCCTCAGTGTCAGGGCACAT and Fam-labeled CAC-CAAGTTCCTCATGCTCTGTTTGG5G, and primers for glyceraldehyde-3-phosphate dehydrogenase were GTGCAGGAGGCATTGCTGAT and Fam-labeled GACGTATGCTGGCGCTGAGTACG5C. Complimentary DNA was generated using the Ambion Retroscript kit (Ambion, Austin, TX) with the Invitrogen Superscript Reverse Transcriptase. The results were analyzed using the I-cycler software, setting a user-defined baseline from 2 to 15 cycles and a user-defined threshold of 50. Values were then

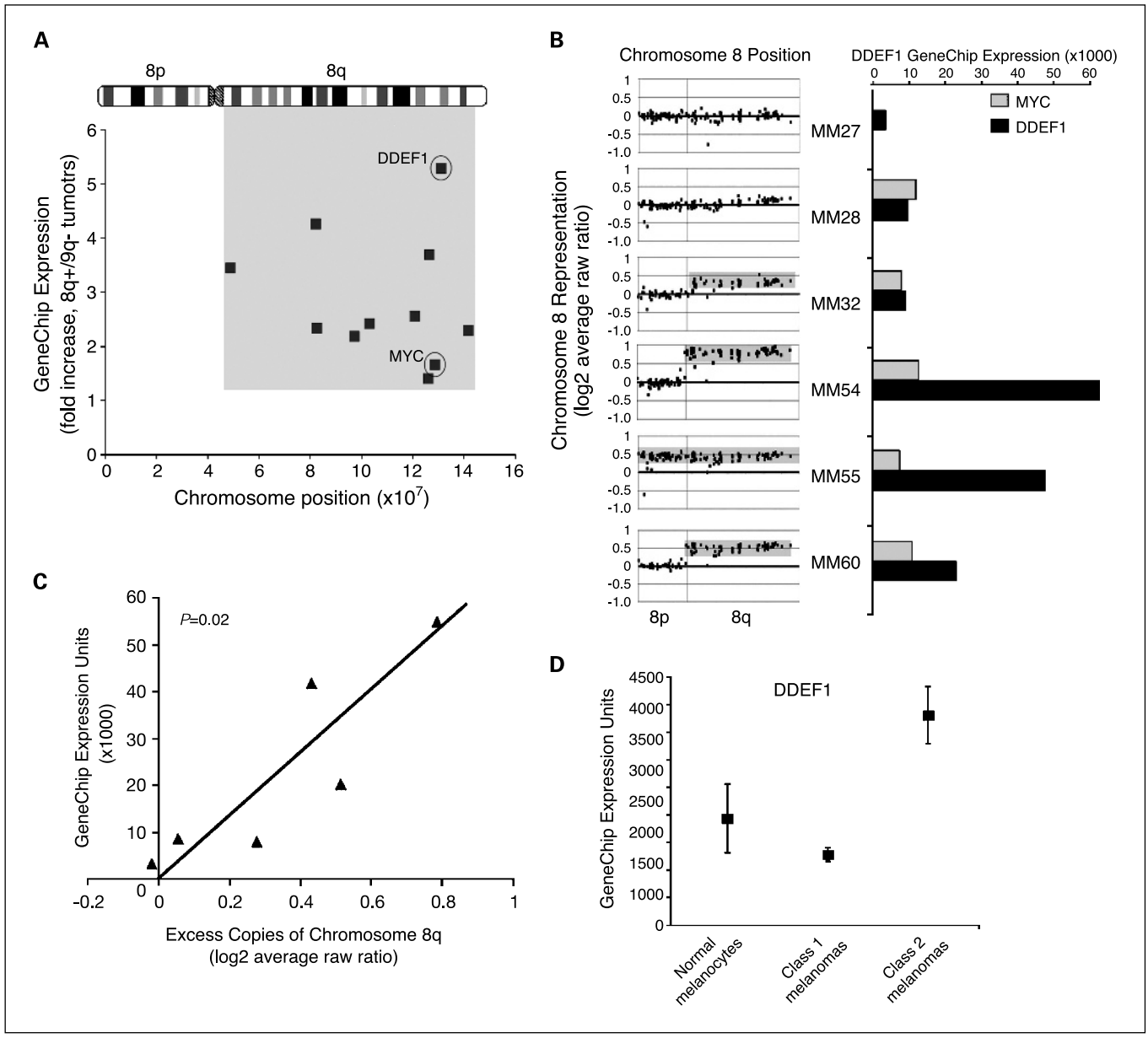

Fig. 1. Gene expression profile analysis of chromosome 8q in uveal melanoma. *A,* highlighted region of chromosome 8q, which is commonly amplified in uveal melanoma, showing microarray gene expression profile versus chromosomal position. Fold increase in expression was measured as mean expression in 8q+ tumors / 8q diploid tumors. *DDEF1* and *MYC* are both noted. *B,* CGH analysis of chromosome 8 in six uveal melanomas. The DNA copy number for each locus was measured as the log 2 average raw ratio, with normal 2N = 0, as previously described (7). Bar graph shows microarray expression of *DDEF1* and *MYC* in the corresponding tumors. *C,* scatter plot showing correlation between chromosome 8q copy number (measured as the mean of the log 2 average raw ratio for all 8q genes) and DDEF1 microarray mRNA expression. *D,* graphic depiction of mean microarray DDEF1 mRNA expression in normal uveal melanocytes and class 1 and class 2 uveal melanomas. Bars, SE.

normalized to glyceraldehyde-3-phosphate dehydrogenase for sample comparison.

Quantitative immunohistochemistry. Formalin-fixed, paraffin-embedded sections from 17 uveal melanomas were immunostained with a 1:500 dilution of anti-DDEF1 antibody (gift from Dr. Paul Randazzo, Division of Basic Sciences, National Cancer Institute, Bethesda, MA) using the streptavidin-biotin method with the Vector ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) as previously described (8). Blue stain was used to eliminate confusion with melanoma-associated brown pigment. Nuclear fast red was used for counterstain. Images were obtained at ×40 magnification and processed in a standardized manner to eliminate red counterstain using Adobe Photoshop software (Adobe, San Jose, CA). Resulting images were analyzed in a masked fashion with ImageJ software (available at http://rsb.info.nih.gov/ij) using the polygon tool and measure function to determine the average intensity per unit area. Cytoplasmic measurements were obtained from 10 random areas and 5 random background fields derived from four separate images. After subtracting the mean background measurement, the mean intensity and SE were calculated for the tumor cells.

Wound-healing assay. Mel202 uveal melanoma cells (maintained in RPMI 1640 10% fetal bovine serum) express low levels of endogenous DDEF1 and cluster with low-grade (class 1) uveal melanomas in our gene expression profile (data not shown). These cells were transfected with a DDEF1 mRNA expression vector or a control empty vector, both in pcDNA3.1neo (gifts of Dr. Thomas Roberts, Department of Cancer Biology, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA) using Effectene (Qiagen) according to the instructions of the manufacturer. To measure cell motility in these transfected cells, we used the wound-healing assay that is widely accepted for this purpose (9). Selection was initiated 24 hours after transfection with G418 (Sigma, St. Louis, MO). Forty-eight hours later, cells were replated at 2×10^5/35-mm dish and grown for 24 hours. Then, a 500-μm-wide "wound" was made on each p35 with a sterile plastic loop. Three digital photos were taken of each wound at 0, 24, 48, and 72 hours. The plates were marked to ensure consistent photodocumentation. Using the ImageJ software, the area of each wound was calculated at each time point. The experiment was conducted in triplicate.

Results

Microarray gene expression and comparative genomic hybridization analysis. To identify candidate oncogenes within the amplified region of chromosome 8q in uveal melanoma, we wished to identify genes in this region that are overexpressed in 8q+ tumors (those with excess representation of chromosome 8q). Microarray gene expression analysis and microarray CGH were both done in six primary, uncultured uveal melanomas. Four of these tumors (MM32, MM54, MM55, and MM60) were found to exhibit varying levels of chromosome 8q overrepresentation (defined as log 2 average raw ratio > |0.5|), whereas the other two (MM27 and MM28) had a normal diploid allocation for this region. Fold overexpression (mean expression of 8q+ tumors / 8q diploid tumors) was plotted against chromosomal position. Using this expression profile, the most overexpressed gene on 8q was *DDEF1*, located at chromosome 8q24.21 (Fig. 1A). When *DDEF1* was compared with *MYC*,

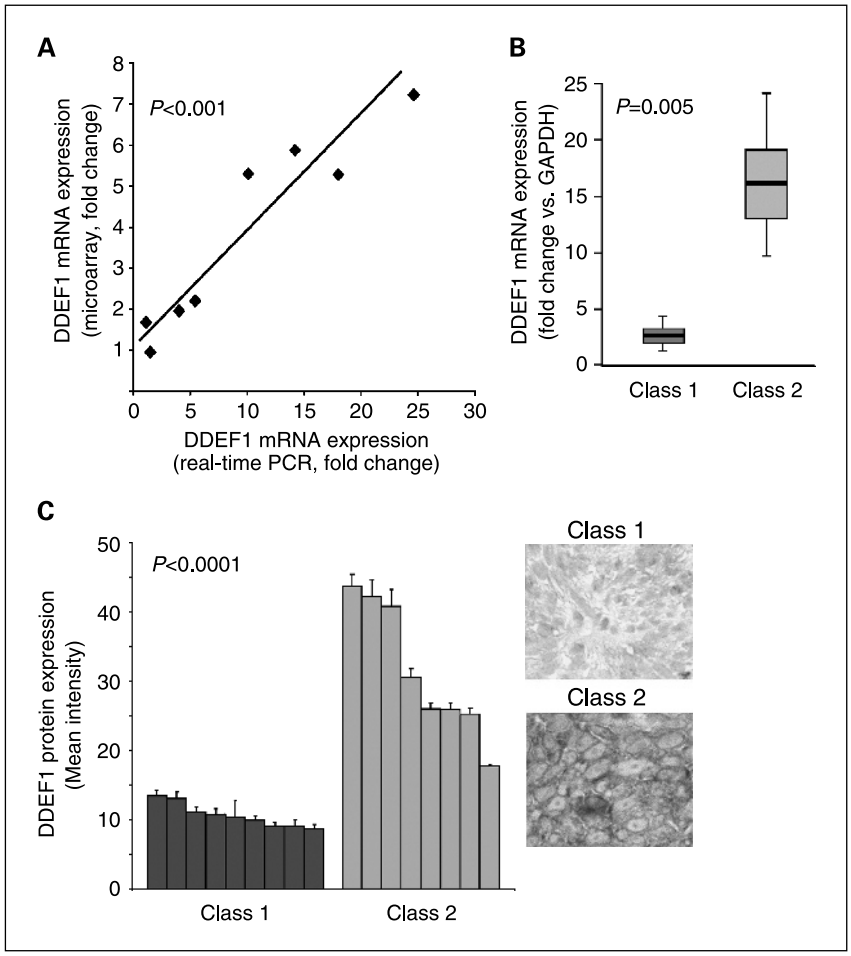

Fig. 2. Validation of DDEF1 microarray expression. *A,* scatter plot depicting the correlation between microarray and real-time PCR gene expression measurements for DDEF1 mRNA in eight primary uveal melanomas. *B,* comparison of mean DDEF1 mRNA expression (measured by real-time PCR) in class 1 versus class 2 uveal melanomas (four tumors in each class). Boxes, SE; bars, range of expression values; heavy bands, mean expression. *C,* comparison of DDEF1 protein expression in class 1 (*dark columns*) versus class 2 (*light columns*) primary uveal melanomas measured by quantitative immunohistochemistry. Each column represents an individual tumor. Right, representative immunohistochemical staining of class 1 and class 2 melanomas immunostained with anti-DDEF1 antibody (positive staining is dark; original magnification, ×40). Bars, SE.

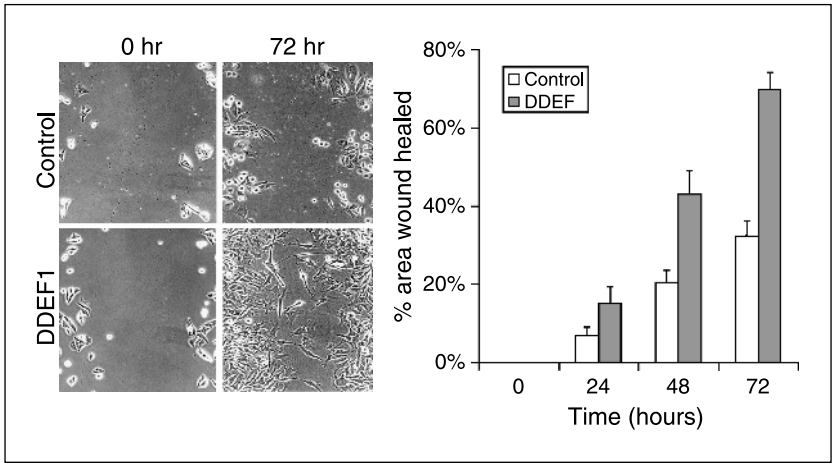

Fig. 3. Effect on cell motility of DDEF1 in uveal melanoma cells. Representative example of wound-healing assay at time 0 and 72 hours (*left*) in Mel202 class 1 uveal melanoma cells stably expressing DDEF1 or control empty vector. Bar graph summarizing wound assay results (*right*). Columns, percentage of area of wound repopulated; bars, SE.

which has been previously implicated as an oncogene in uveal melanoma, DDEF1 mRNA expression was increased in 8q+ tumors, whereas MYC expression remained unchanged (Fig. 1B). Further, when the number of copies of 8q was estimated as the mean of the log 2 average raw ratio for each marker on 8q, *DDEF1* overexpression correlated strongly with 8q copy number (Pearson correlation, $r = 0.87$, $P = 0.02$; Fig. 1C).

We previously reported a novel molecular classification of uveal melanoma based on gene expression profile (5). This classification showed high predictive value for metastasis and death: low-grade (class 1) tumors rarely metastasized, whereas high-grade (class 2) tumors frequently metastasized. We used 25 primary uveal melanomas from this study to analyze the correlation between *DDEF1* expression and the molecular classification. Interestingly, DDEF1 mRNA was expressed at similar levels in normal uveal melanocytes and class 1 melanomas, but it was expressed at significantly higher levels in the class 2 tumors (Fig. 1D). This finding suggests that up-regulation of *DDEF1* may occur later during tumor progression rather than during the early events in malignant transformation, which would be consistent with the later timing of 8q amplification (10).

Quantitative PCR and immunohistochemistry. To validate these findings, we analyzed DDEF1 mRNA expression in uncultured tumor tissue from four class 1 tumors and four class 2 tumors using real-time PCR. DDEF1 mRNA expression by PCR strongly correlated with microarray results (Pearson correlation, $r = 0.93$, $P < 0.001$; Fig. 2A) and exhibited a mean *DDEF1* up-regulation of 16-fold in class 2 tumors ($P = 0.005$; Fig. 2B). We then used 17 paraffin-embedded tumors to evaluate DDEF1 protein expression using quantitative immunohistochemistry. There was a strong correlation between microarray mRNA expression and cytoplasmic immunostaining intensity (Pearson coefficient, $r = 0.81$, $P < 0.0001$), and the DDEF1 protein was significantly up-regulated in class 2 tumors ($P < 0.0001$; Fig. 2C).

Cell motility. Because *DDEF1* has been shown to play a role in cell motility (11, 12), we wished to study the effect of DDEF1 overexpression on the motility of uveal melanoma cells. We expressed ectopic DDEF1 (or a control empty vector) in Mel202 class 1 uveal melanoma cells and measured the ability of the cells to repopulate a scratch "wound" as an indication of cell motility, as previously described (9). DDEF1-overexpressing cells repopulated the wound more rapidly than control cells ($P < 0.0001$; Fig. 3).

Discussion

Amplification of chromosome 8q is one of the most common genetic abnormalities in uveal melanoma and is strongly associated with metastatic death (2, 3). However, a pathogenetically relevant oncogene in this region has not been identified. MYC is located at 8q24 and is located within the region that is amplified in uveal melanomas (3). However, MYC expression does not correlate with metastatic death (4), and we show here that MYC is not up-regulated in high-grade melanomas. In contrast, *DDEF1* exhibits several features consistent with its role as an oncogene in uveal melanoma: it is located in the amplified region of chromosome 8q; its expression correlates with the gain of 8q; and it is significantly overexpressed at both the mRNA and protein levels in high-grade uveal melanomas. Furthermore, expression of *DDEF1* in low-grade melanoma cells causes increased cell motility consistent with progression to a high-grade phenotype.

Development and differentiation enhancing factor 1 (*DDEF1*), also known as *A*rf-GAP containing *S*H3, *a*nkyrin repeats and *p*leckstrin domain (*ASAP1*), is an ADP ribosylation factor-GTPase activating protein that interacts with signal transduction proteins involved in growth and differentiation—such as SRK, FAK, phosphatidylinositol 4,5-biphosphate, and CRK—and regulates actin cytoskeletal remodeling that is necessary for cell motility (12–15). Cell motility involves an inhibition of cell spreading followed by extension of peripheral elastic lamellae in the direction of locomotion, which requires the continual remodeling of the actin cytoskeleton and assembly/disassembly of focal adhesions at the leading and trailing edges, respectively, of the motile cell (16). DDEF1 protein localizes to newly forming focal complexes at the cell periphery and regulates these cyclical changes in the cytoskeleton and focal adhesions (13). Overexpression of DDEF1 protein disrupts focal adhesion turnover, thereby blocking cell spreading and promoting cell motility (17). Hence, oncogenic up-regulation of *DDEF1* may explain, at least in part, the increased invasion and metastatic potential of high-grade uveal melanoma.

These findings may provide new insights into uveal melanoma pathogenesis and suggest that DDEF1 may be an effective therapeutic target in high-grade uveal melanomas and other cancers. Further, DDEF1 may be a useful diagnostic marker to identify uveal melanoma patients at high risk of metastasis.

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