NBS1 Expression as a Prognostic Marker in Uveal Melanoma

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ABSTRACT

Purpose: **Up to half of uveal melanoma patients die of metastatic disease. Treatment of the primary eye tumor does not improve survival in high-risk patients due to occult micrometastatic disease, which is present at the time of eye tumor diagnosis but is not detected and treated until months to years later. Here, we use microarray gene expression data to identify a new prognostic marker.**

***Experimental Design*: Microarray gene expression profiles were analyzed in 25 primary uveal melanomas. Tumors were ranked by support vector machine (SVM) and by cytologic severity. Nbs1 protein expression was assessed by quantitative immunohistochemistry in 49 primary uveal melanomas. Survival was assessed using Kaplan-Meier life-table analysis.**

Results: **Expression of the Nijmegen breakage syndrome (*NBS1*) gene correlated strongly with SVM and cytologic tumor rankings ($P < 0.0001$). Further, immunohistochemistry expression of the Nbs1 protein correlated strongly with both SVM and cytologic rankings ($P < 0.0001$). The 6-year actuarial survival was 100% in patients with low immunohistochemistry expression of Nbs1 and 22% in those with high Nbs1 expression ($P = 0.01$).**

Conclusions: NBS1 **is a strong predictor of uveal melanoma survival and potentially could be used as a clinical marker for guiding clinical management.**

INTRODUCTION

Uveal melanoma is the most common cancer of the eye and the second most common form of melanoma (1). Up to half of uveal melanoma patients die of metastatic disease, and in most of these high-risk patients, occult micrometastatic disease is thought to be present (but undetectable) at the time the primary eye tumor is diagnosed and treated (2). Unknowingly, systemic treatment is routinely withheld from these high-risk patients until overt metastatic disease is detected, usually 2 to 5 years later, by which time available therapies are usually ineffective. Delayed detection of micrometastasis may account for the lack of improved survival conferred by treatment of the primary eye tumor and the abysmal track record for treating metastatic uveal melanoma (3).

These findings suggest that a rational strategy for improving survival in uveal melanoma patients would be to identify at the time of diagnosis of the primary eye tumor those patients who are at high risk of metastasis, and to treat those patients prophylactically with adjuvant systemic therapy. Numerous clinical, pathologic, and cytogenetic prognostic factors have been evaluated in uveal melanoma (4) but none has proven to be sufficiently accurate and/or feasible for routine clinical use. To address this problem, we recently developed a novel prognostic assay for uveal melanoma based on gene expression profiling (5). Remarkably, these tumors clustered naturally into two distinct classes that correlated strongly with metastatic risk.

Whereas our previous study was designed to identify patterns of gene expression that differentiated tumor classes, the objective of the present study was to identify a highly discriminating gene of which expression could potentially stand alone as a prognostic marker (i.e., not part of a gene profile or "signature"). The protein product of the Nijmegen breakage syndrome 1 (*NBS1*) gene, which plays a critical role in double strand DNA damage repair, was identified as a marker that correlated strongly with tumor severity and metastatic death. These findings suggest that *NBS1* may be a clinically useful prognostic marker in uveal melanoma.

MATERIALS AND METHODS

Microarray Gene Expression Analysis. These studies were done in accordance with a protocol approved by the Washington University Institutional Review Board. Microarray gene expression analysis was done using a previously reported data set from 25 primary, uncultured uveal melanomas (5). Briefly, total RNA was obtained from primary uncultured uveal melanomas using TRIzol (Invitrogen, Carlsbad, CA) and purified using RNeasy kits (Qiagen, Valencia, CA) according to instructions of the manufacturers. In addition, we obtained RNA in a similar fashion from three low passage normal uveal melanocyte cell lines developed in our laboratory. RNA quality was assessed on the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Complementary DNA was generated from total RNA by reverse transcription and subjected to linear amplification and *in vitro* transcription to generate biotinylated cRNA targets, which were hybridized to Affymetrix Hu133A and B GeneChips (Affymetrix, Santa Clara, CA) according to the protocols of the manufacturer. Chips were checked for quality assurance variables and normalized for mean overall expression, and probe sets were analyzed for significance using Affymetrix software. Gene expression values were subjected to $\log_{10}$ transformation and scalar normalization by the minimum value. Analysis of microarray expression results was done by Pearson correlation and Student's t test as appropriate. Principal component

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analysis was done using Spotfire DecisionSite 7.0 software, and support vector machine (SVM) was done using GIST software available at http://microarray.cpmc.columbia.edu/gist.

Quantitative PCR. RNA was isolated from eight primary uncultured uveal melanomas using TRIzol (Invitrogen) and purified using RNeasy kits (Qiagen) according to the instructions of the manufacturer. Complimentary DNA was generated for PCR analysis using RETROscript kit (Ambion, Austin, TX) according to the instructions of the manufacturer. Real-time PCR was done using the Invitrogen Lux primer system (Invitrogen) following the protocol published by the manufacturer for the Biorad I-cycler (Bio-Rad Laboratories, Hercules, CA). Primer sets for *NBS1* were CTGTGGACGACCCGATGAG and Fam-labeled GACTCCACGCACCCACTGTAAAGGAG5C. Primer sets for GAPDH were GTGCAGGAGGCATTGCTGAT and Fam-labeled GACGTATGCTGGCGCTGAGTACG5C. The 20 μL reaction was diluted to 100 μL in diethylpyrocarbonate-treated water, and 1 μL was used in each PCR reaction. Data were analyzed using I-cycler software, setting a user-defined baseline from 2 to 15 cycles and a user-defined threshold of 50. Values were then normalized to GAPDH.

Immunohistochemical Staining. Immunohistochemistry was done on 49 primary uveal melanomas using the streptavidin-biotin method with the Vector ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and blue stain to avoid confusion with brown melanin pigment, as previously described (6). Nuclear fast red was used for counterstain. Four-micron sections were obtained, deparaffinized, rehydrated with ethanol, and treated with 0.3% hydrogen peroxide and methanol to inhibit endogenous peroxidase activity. Heat-induced antigen retrieval was done using microwave treatment in citrate buffer. Anti-Nbs1 antibody (Cell Signaling Technology, Beverly, MA) was applied at a dilution of 1:75 at 4°C overnight. The secondary antibody alone was used as a negative control.

Immunohistochemistry Quantitation and Statistical Analysis. Images from immunostained tumor sections were obtained on a microscope-mounted camera at 400× 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 straight line tool and the plot profile function to calculate nuclear staining intensities. Measurements were obtained from 25 random nuclei and 4 random background fields from 4 separate fields per tumor, for a total of 16 background measurements and 100 nuclear measurements per tumor. After subtracting the mean background measurement, the mean intensities and SEs were calculated. Student's t test and Pearson correlation coefficients were used as appropriate to compare Nbs1 immunostaining intensity with clinical and pathologic features.

Survival Analysis. Survival analysis was done on 18 patients with follow-up of at least 5 months using Kaplan-Meier life table analysis (MedCalc software, version 7.2.0.2, http://www.medcalc.be). Survival was defined as the elapsed interval from the date of enucleation to the date of last follow-up or death, all of which were due to melanoma metastasis.

RESULTS

Mathematical Ranking of Tumor Samples. In our original microarray gene expression analysis, Affymetrix GeneChip expression values were obtained from ~44,69 probe sets in 25 primary uncultured uveal melanomas (5). In the present study, where our primary goal is to identify a highly discriminating marker for high-risk tumors, we did several additional enrichment procedures on this data set. First, the probe set significance threshold (using Affymetrix software) was lowered from $P < 0.005$ to $P < 0.0005$. This process eliminated all but the 1,249 of the most significant probe sets. Second, we used mathematical tumor rankings (rather than more subjective cytologic ranking) to identify correlations with gene expression. This mathematical ranking was based on SVM, which is a supervised training method that generates a discriminant score reflecting the proximity of each tumor to a hyperplane separating the two tumor classes (7). We generated SVM models using various combinations of samples and genes as training sets, and all of them yielded similar results (data not shown). The discriminant scores obtained from a 12 sample/3 gene training set were used in subsequent correlative analysis using Pearson coefficients. The two genes that showed the strongest association with SVM discriminant scores were *TRAM1* ($r = 0.795$, $P < 0.0001$) and *NBS1* ($r = 0.751$, $P < 0.0001$). Whereas *TRAM1* encodes a poorly understood endoplasmic reticulum protein, *NBS1* is a well-characterized gene that plays a critical role in development, DNA damage repair, and carcinogenesis. Thus, our subsequent studies focused on *NBS1* (Fig. 1*A*).

Categorical Analysis of Tumor Class Labels. We previously showed that primary uveal melanomas could be grouped into two classes based on gene expression profile, and that these classes strongly predicted metastatic death (5). Analysis of *NBS1* microarray gene expression as a function of categorical class label (i.e., class 1 or class 2) revealed a highly significant difference in expression between the two groups ($P < 0.0001$; Fig. 1*B*). Interestingly, *NBS1* expression was not increased in class 1 melanomas compared with normal uveal melanocytes (Fig. 1*B*), suggesting that *NBS1* up-regulation may only occur later in tumor progression.

The top 20 discriminating genes identified by Pearson correlation coefficients were compared for mean expression in normal uveal melanocytes, class 1 and class 2 melanomas (Supplementary Table). Interestingly, none of these genes were up-regulated ≥1.5-fold in class 1 melanomas versus normal melanocytes, suggesting that the gene selection criteria used in this study were more likely to identify markers of later melanoma progression rather than early melanoma formation.

To validate the microarray results, we examined *NBS1* mRNA expression using real-time PCR in eight of the tumor samples. There was a strong correlation between microarray and PCR expression values ($r = 0.85$; $P = 0.008$), and the difference in expression between class 1 and class 2 tumors was significant ($P = 0.01$; Fig. 1*C*).

Quantitative Immunohistochemistry. Owing to immunohistochemistry being more practical for routine clinical testing, we wished to determine whether detection of Nbs1 protein expression by immunohistochemistry would correlate with the aforementioned measures of tumor severity as well as

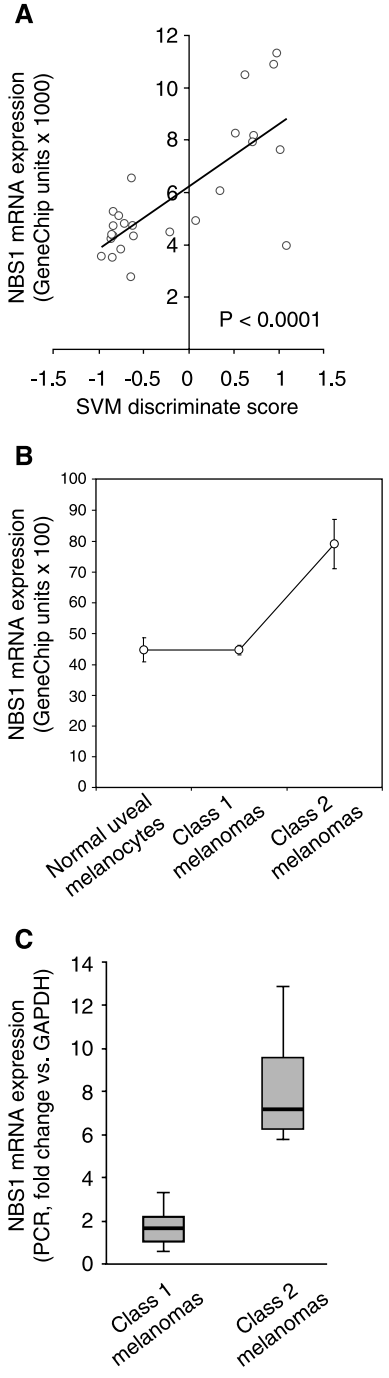


Fig. 1 *NBS1* mRNA expression is up-regulated in high-grade uveal melanomas. Supervised training and cytologic ranking models reveal correlation between tumor severity and *NBS1* microarray gene expression. *A,* scatter plot demonstrating correlation between *NBS1* gene expression (as measured by Affymetrix GeneChip arrays) and SVM score, which assigns tumors a discriminant score reflecting the proximity of each tumor to a hyperplane separating two tumor classes. *B,* mean microarray GeneChip expression of *NBS1* in normal uveal melanocytes and class 1 and class 2 uveal melanomas. *Bars,* SE. *C*, mean real-time PCR gene expression of *NBS1* in class 1 versus class 2 tumors. Gene expression is represented as fold increase compared with GAPDH. *Vertical bars,* range of values; *horizontal bars,* mean; *boxes,* SE.

clinical prognostic factors. Quantitative immunohistochemistry for Nbs1 was done on 49 paraffin-embedded, primary uveal melanomas, including 18 of the tumors analyzed for microarray gene expression above. Nuclear immunostaining for Nbs1 varied from weak to very strong (Fig. 2*A*), and staining intensity correlated strongly with both SVM discriminant score and cytologic rank (r = 0.908 and 0.889, respectively; Fig. 2*B* and *C*). Similarly, Nbs1 immunostaining intensity was strongly associated with molecular class label ($P < 0.0001$; Fig. 2*D*). There was no significant association with clinical prognostic factors such as patient age, gender, tumor size, or anterior tumor location (data not shown).

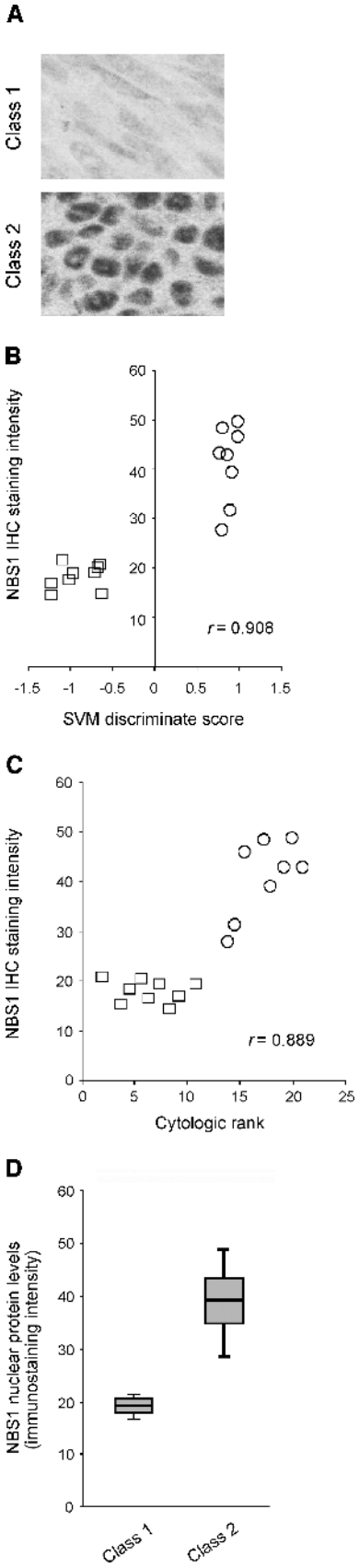
Survival Analysis. To determine whether Nbs1 immunostaining may correlate with metastatic death, we studied the survival of 18 patients with follow-up of at least 6 months. We divided the patients into equal groups ("low Nbs1" and "high Nbs1") around the median Nbs1 expression value. Four deaths occurred, all in the high *Nbs1* group. By Kaplan-Meier life table analysis, the 6-year actuarial survival was 100% for the low Nbs1 group and 22% for the high Nbs1 group (P = 0.01; Fig. 3).

DISCUSSION

These studies identify a novel association between *NBS1* and metastatic death from uveal melanoma. *NBS1* is up-regulated at both the mRNA and protein levels in class 2 (poor prognosis) primary uveal melanomas relative to class 1 (good prognosis) melanomas. The fact that *NBS1* expression is similar in class 1 tumors and normal uveal melanocytes suggests that up-regulation of *NBS1* may be a late event in melanoma progression. Owing to immunostaining for the Nbs1 protein using standard immunohistochemistry techniques showing a strong association with tumor severity and metastatic death, this marker could become a clinically useful prognostic tool that could be used at the time of initial treatment of the primary eye tumor to identify patients that may benefit from prophylactic adjuvant systemic therapy.

The product of the *NBS1* gene, nibrin, is a part of the *MRE11/RAD50/NBS1* complex that is involved in DNA double-strand break repair; phosphorylation of nibrin by ATM in response to ionizing radiation triggers a DNA damage-dependent S-phase checkpoint that inhibits DNA replication (8, 9). *NBS1* is defective in the autosomal recessive Nijmegen breakage syndrome, characterized by growth retardation, microcephaly, immunodeficiency, radiosensitivity, and tumor predisposition in both humans and mice (10). Whereas loss of *NBS1* causes chromosomal instability and radiation sensitivity, uveal melanomas are noted for their relative lack of genomic instability and their extreme resistance to radiation therapy (11, 12), suggesting that *NBS1* overexpression potentially could contribute to this phenotype through more efficient repair of DNA damage. Interestingly, *NBS1* is also a direct transcriptional target of the *Myc* oncogene, which is often amplified in uveal melanomas (13), and links *Myc* to the DNA double strand break repair pathway (14). Interestingly, *NBS1* was the only gene among the top 100 discriminating genes identified by Pearson correlation that is involved in DNA damage repair according to Gene Ontology (data not shown).

Several other studies of microarray gene expression have been done in melanoma cell lines (15, 16). Clark et al. (15)



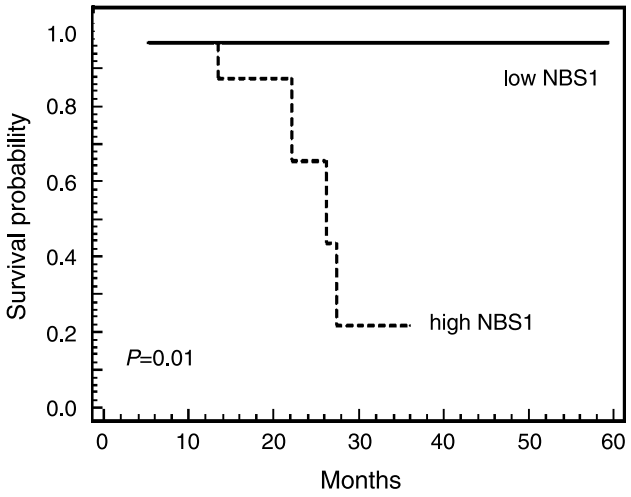


Fig. 3 Kaplan-Meier life-table survival analysis of 18 uveal melanoma patients stratified by Nbs1 protein nuclear immunostaining.

identified the small GTPase RhoC as a metastasis enhancer. Bittner et al. (16) found that gene expression profiling could distinguish melanoma cell lines by their ability to spread and migrate. This article included several uveal melanoma cells lines that revealed integrin β1, integrin β3, integrin α1, syndecan 4, vinculin, and fibronectin as discriminating genes, suggesting a role for focal contacts in modulating melanoma cell motility. This group further identified WNT5A as the best *in vitro* determinant of invasive behavior (17). Interestingly, none of these were among the top discriminating genes in the present study nor in our prior article (5). In contrast, there is considerable overlap between our gene set and that published by Tschentscher et al. (18) who also used primary, uncultured uveal melanomas like we did. Thus, it is possible that the differences in data derived from these various studies can be explained, at least in part, by the use of cutaneous versus uveal melanomas and primary uncultured tumor tissue versus cultured cell lines.

These findings provide evidence for a potentially useful clinical prognostic marker for metastatic death in uveal melanoma. This study differs from our previously published microarray analysis both in objective and approach to data analyses. Whereas the previous study used both unsupervised and supervised techniques to identify gene expression patterns that correlated with metastatic risk (5), this study was designed to identify a highly significant marker of which expression could stand alone as a prognostic marker. The findings of this small

Fig. 2 Quantitative immunohistochemical analysis of Nbs1 protein expression. *A,* representative class 1 (*top*) and class 2 (*bottom*) uveal melanomas immunostained with anti-Nbs1 antibody (dark nuclear staining). Original magnification, ×100. *B,* scatter plot demonstrating correlation between quantitative immunostaining for Nbs1 protein and SVM score (see Fig. 1*B* legend and text for details). *Squares,* class 1 tumors; *circles,* class 2 tumors. *C,* scatter plot demonstrating correlation between quantitative immunostaining for Nbs1 protein and cytologic rank. *Squares,* class 1 tumors; *circles,* class 2 tumors. *D,* quantitative analysis of Nbs1 nuclear immunostaining in class 1 and class 2 tumors. *Vertical bars,* range of values; *horizontal bars,* mean; *boxes,* SE.

pilot study need to be validated in a larger patient cohort. Owing to *NBS1* expression exhibiting prognostic accuracy equal to the entire gene expression signature in this small study, it is possible that immunohistochemical staining for Nbs1 alone would be sufficient for routine clinical testing. Further work is indicated to explore the functional significance of these observations, which could reveal new mechanistic insights and therapeutic targets.

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