Loss of Heterozygosity of Chromosome 3 Detected with Single Nucleotide Polymorphisms Is Superior to Monosomy 3 for Predicting Metastasis in Uveal Melanoma

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Abstract

Purpose: Loss of chromosome 3 is strongly associated with metastasis in uveal melanoma and has been proposed as the basis for clinical prognostic testing. It is not known whether techniques that identify loss of heterozygosity for chromosome 3 predict metastasis more accurately than those that detect only numerical loss of chromosome 3 (monosomy 3).

Experimental Design: Fifty-three uveal melanomas were analyzed by 28 single nucleotide polymorphisms (SNP) across chromosome 3. SNP was compared with fluorescence *in situ* hybridization (FISH) and array-based comparative genomic hybridization (aCGH) for metastasis prediction by sensitivity, specificity, and Kaplan-Meier survival analysis, using our validated gene expression-based classifier as a reference standard.

Results: By Kaplan-Meier analysis, only the gene expression-based classifier ($P = 0.001$) and SNP-based detection of loss of heterozygosity for chromosome 3 ($P = 0.04$) were significantly associated with metastasis. Sensitivity and specificity were 95.2% and 80.8%, respectively, for SNP, 77.8% and 64.7%, respectively, for FISH, and 85.0% and 72.0%, respectively, for aCGH. Isodisomy 3 was identified by SNP but undetected by aCGH and FISH in three tumors.

Conclusions: Prognostic tests based on SNP platforms, which detect both chromosomal homologues and their subregions, may be superior to techniques that only detect changes in chromosome number. These observations could have important implications for efforts to detect genetic alterations in cancer genomes with CGH-based approaches.

Uveal melanoma is the most common primary cancer of the eye and has a strong predilection for hematogenous metastasis, particularly to the liver (1). Up to half of uveal melanoma patients develop metastasis with a median time of 2.4 years from ocular diagnosis, usually leading to death within a few months (2). This has lead some investigators to propose that high-risk patients should be treated with prophylactic systemic therapy (3). However, an accurate prognostic classifier for identifying high-risk patients who may benefit from prophylactic therapy has not been validated.

Many clinical and pathologic features have been associated with metastatic disease, but none of these has been shown to have adequate sensitivity and specificity for making personalized clinical decisions. Monosomy 3, detected by cytogenetic analysis, spectral karyotyping, fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and other techniques, may be more accurate than clinical and pathologic features and has been adopted as a molecular prognostic marker in many centers (4–12). More recently, two distinct molecular subgroups were identified by gene expression profiling that correlate strongly with metastatic risk (13, 14). Tumors with the class 1 expression signature had a low risk, and those with the class 2 signature had a high risk of metastasis. Although there was a strong association between the class 2 signature and monosomy 3, the gene expression-based classifier was superior in prognostic accuracy to monosomy 3 (13). Elucidating the reasons for this superiority is important, not only from a biological standpoint, but also from a practical one. Although gene expression profiling may be more accurate, DNA-based chromosome 3 testing may be necessary where high quality RNA is not available such as in tumors that are partially necrotic or have been embedded in paraffin.

The inferior performance of monosomy 3 compared with the gene expression classifier may be due, at least in part, to technical limitations in the methods that have been used to detect monosomy 3. Single nucleotide polymorphism (SNP)

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analysis has emerged as a promising method for detecting chromosome copy number changes and loss of heterozygosity (LOH) in cancer (15, 16). We have developed a high-throughput, mass spectrometry–based assay to interrogate highly polymorphic SNPs distributed across chromosome 3. In this study, the prognostic accuracy of the SNP assay was compared with traditional methods for measuring monosomy 3, including array CGH (aCGH) and FISH. Detection of LOH by SNP was superior to aCGH and FISH, due in part to its ability to detect isodisomy 3. These findings may have important implications for efforts to identify genetic rearrangements in cancer.

Materials and Methods

Preparation of DNA. This study was approved by the Human Studies Committee at Washington University. Informed consent was obtained from each subject. Tumor tissue from uveal melanoma patients was obtained at the time of eye removal, immediately snap frozen in the operating room, and stored at -80°C until DNA preparation. Genomic DNA was prepared using the Wizard Genomic DNA purification kit (Promega). Normal DNA was purified from peripheral blood samples using the PureGene DNA Purification System Blood kit (Gentra Systems).

SNP analysis. SNPs were selected from the Ensembl Homo sapiens SNPs database (based on dbSNP 126, HGVbase 15, TSC 1, and Affymetrix GeneChip Mapping Array) with minor allele frequencies >0.4 in the European population (which describes all patients in the study) at approximate intervals of 6 ± 0.5 Mb across the euchromatic regions of chromosome 3. Sequenom SpectroDESIGNER software was used for the design of primers to permit genotyping in multiplex fashion ("IPLEX" software). We selected 35 SNPs that met these selection criteria and tested them on tumor DNA samples with known genotypes. Seven SNPs were eliminated due to poor primer performance or lower than expected minor allele frequency. The resulting 28 SNPs were included in the LOH assay. Reference IDs for SNPs that were included and excluded from the final assay are available on request. SNP-mass spectrometry-genotyping (16) was done with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry by the Division of Human Genetics Genotyping core facility[4] using the Sequenom MassARRAY system. Allele calls and confidence scores were made by Sequenom software. For this initial analysis, only high confidence calls were included. Matching normal DNA was analyzed in 22 cases to verify the accuracy of allele calls.

Statistical analysis. Fisher's exact test was used to assess the significance of association between two categorical variables. Kaplan-Meier analysis was used to assess time-dependent association with metastasis for categorical variables. Sensitivity, specificity, likelihood ratios, and predictive values were assessed using metastasis as the primary end point and gene expression-based classification (class 1 or class 2) as a surrogate end point in metastasis-free patients with <5 years follow-up. This surrogate end point was selected based on the high predictive accuracy of the gene expression classifier (17). All statistical analyses were done using MedCalc software[5] (version 9.0.0.1).

Results

Clinical, pathologic, and molecular features of the 53 uveal melanomas included in this study are summarized in Table 1. SNP analysis was done on all tumor DNA samples and on matching peripheral blood DNA in 22 cases (Fig. 1A-C). Of 2,772 total SNP calls, 77% were scored as high confidence, and only these calls were used for subsequent analyses. The SNP assay revealed retention of heterozygosity for chromosome 3 in 29 tumors and LOH in 24 tumors (Supplementary Table). For unmatched samples, the median number of informative SNPs was 25 (range, 20-28) for retention of heterozygosity tumors and 15 (range, 11-25) for LOH tumors. For matched tumors exhibiting LOH, 65% of the SNPs that were heterozygous in the normal DNA received a 'no call' by the Sequenom software in the tumor DNA. Inspection of the raw spectral data from these cases showed a prominent peak for the homozygous tumor allele and a minor but discernible peak that corresponded to the second allele in the residual normal/germ-line DNA (Fig. 1D), consistent with LOH in the tumor DNA and a small amount of contaminating normal DNA. The unmatched tumor with the fewest informative SNPs was MM66, which exhibited LOH. Based on the allele frequencies for the 11 informative SNPs in MM66, the probability that all would be homozygous in the absence of LOH was 0.0004, thus providing a conservative minimum estimate of significance for all LOH calls.

Most of the tumors in this study were analyzed previously by microarray gene expression profiling, dual-color FISH, and/or aCGH (13, 17, 18). Therefore, we were able to compare the SNP-based LOH assay with these other molecular techniques. Of 42 tumors analyzed by the gene expression-based classifier, 23 were assigned to class 1 and 19 to class 2. Of the 28 tumors analyzed by FISH, 18 were disomy 3 and 10 were monosomy 3. Of the 45 tumors analyzed by aCGH, 26 were disomy 3 and 19 were monosomy 3 (Supplementary Table). By Fisher's exact test, there was a significant association between the SNP assay results and the gene expression-based classifier ($P = 1 \times 10^{-9}$),

Table 1. Summary of clinical and pathologic features

Clinicopathologic factors	Patients (*N* = 53)
Age at diagnosis, mean (range)	60.7 (24-87)
Gender	
Male	33 (62%)
Female	20 (38%)
Eye	
Right	26 (49%)
Left	27 (51%)
Tumor location	
Posterior	30 (57%)
Anterior	23 (43%)
Largest tumor diameter, mm, mean (range)	17.5 (5.4-24)
Tumor thickness, mm, mean (range)	9.8 (2.2-22)
Histopathologic cell type	
Spindle	16 (30%)
Mixed	15 (28%)
Epithelioid	22 (42%)
Histopathologic local invasion	
None	20 (38%)
Intrascleral	18 (34%)
Extrascleral	15 (28%)
Metastasis	13 (25%)
Months from diagnosis to end point, mean (range)	25.4 (1-67)

[4] http://hg.wustl.edu/info/Sequenom.description.html
[5] http://www.medcalc.be

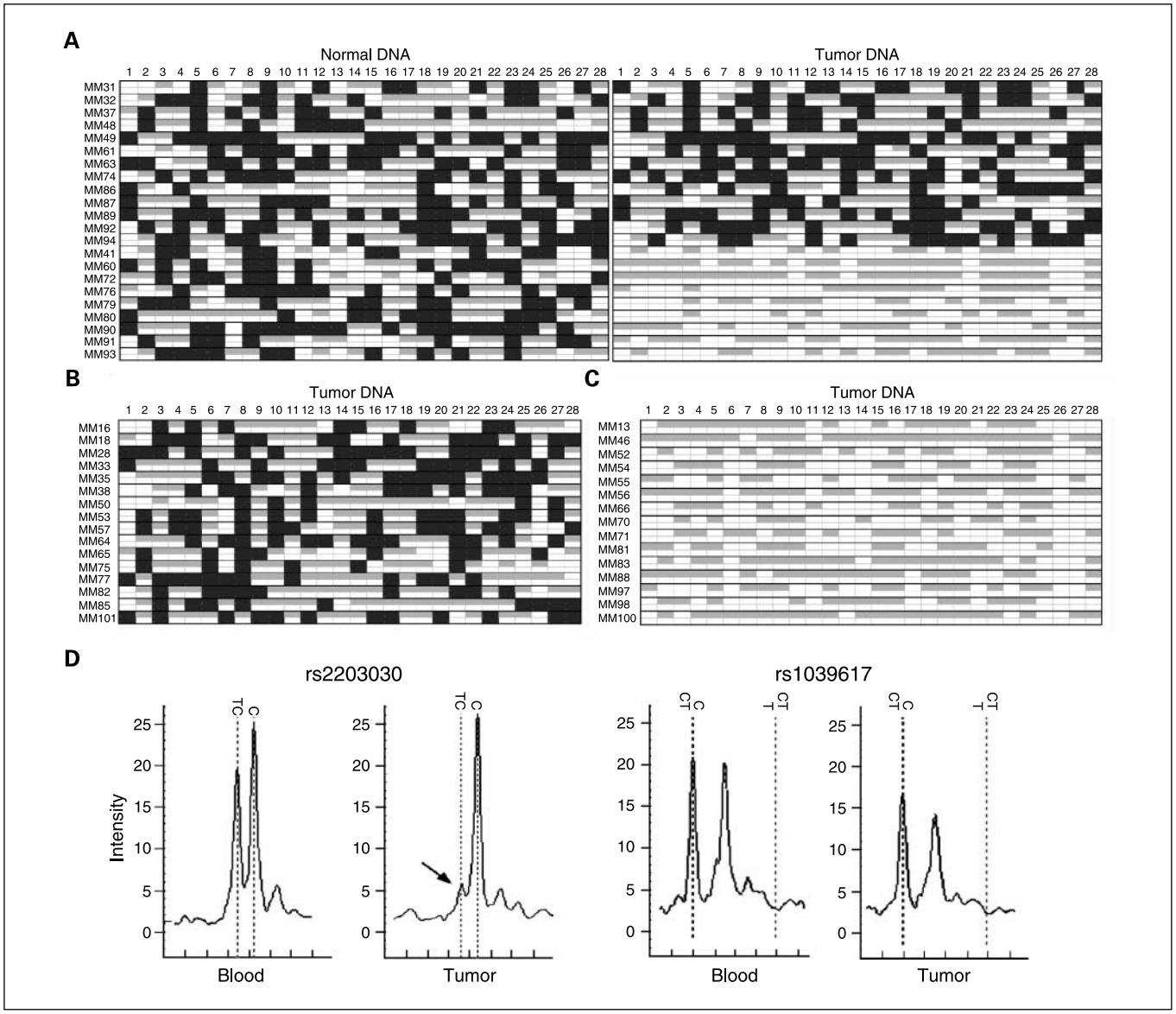

Fig. 1. Summary of SNP results. *A,* matching normal (*left*) and tumor DNA (*right*) in 22 cases. *B,* tumor DNA without matching normal DNA in 16 tumors that show retention of heterozygosity for chromosome 3. *C,* tumor DNA without matching normal DNA in 15 tumors that show LOH. Each pair of rows represents a normal and/or tumor sample. Columns represent SNPs, which are numbered consecutively. Thus, each SNP is represented by two boxes for each normal or tumor sample. For SNPs that showed heterozygosity, both boxes are shaded in black. For SNPs that showed homozygosity, the upper box is shaded in gray. *D,* raw spectral data for two SNPs in tumor and normal DNA from case MM41. The rs2203030 SNP, which received a 'no call' by the Sequenom software, displayed heterozygosity (two peaks of similar intensity) in normal blood DNA but one major peak in the tumor DNA. However, the tumor DNA also contained a minor peak (*arrow*) corresponding to the second allele in the residual normal/germ-line DNA. In contrast, the rs1039617 SNP displayed homozygosity in normal blood DNA and a corresponding single peak in the tumor DNA. Note that the peaks that are not demarcated by a dotted line correspond to unrelated, multiplexed SNPs.

aCGH ($P = 5.8 \times 10^{-7}$), and FISH ($P = 0.016$). Using the gene expression-based classifier as a benchmark, the SNP assay exhibited 1 of 23 (4%) false-negative calls and 1 of 19 (5%) false-positive calls, the aCGH assay 3 of 22 (14%) false-negative calls and 3 of 19 (15%) false-positive calls, and FISH 5 of 17 (29%) false-negative calls and 2 of 9 (22%) false-positive calls. Three of the tumors that were called disomy 3 by aCGH and FISH but class 2 by gene expression profile (MM72, MM81, and MM91) exhibited LOH by SNP, consistent with isodisomy 3. Thus, SNP was concordant with the gene expression classifier in these three cases due to its ability to detect isodisomy 3.

By Kaplan-Meier analysis, only the class 2 gene expression signature ($P = 0.001$) and chromosome 3 LOH detected by SNP ($P = 0.04$) were significantly associated with metastasis (Fig. 2). All three of the tumors with isodisomy 3 also exhibited the class 2 expression signature, and one of the three had developed metastasis at the time of the study.

Sensitivity, specificity, likelihood ratios, and predictive values were calculated for the SNP LOH assay and the two techniques for detecting monosomy 3 (Table 2). To increase the statistical power of this analysis, the primary end point was metastasis, and the secondary end point was the gene expression-based classification because this prognostic test previously was shown

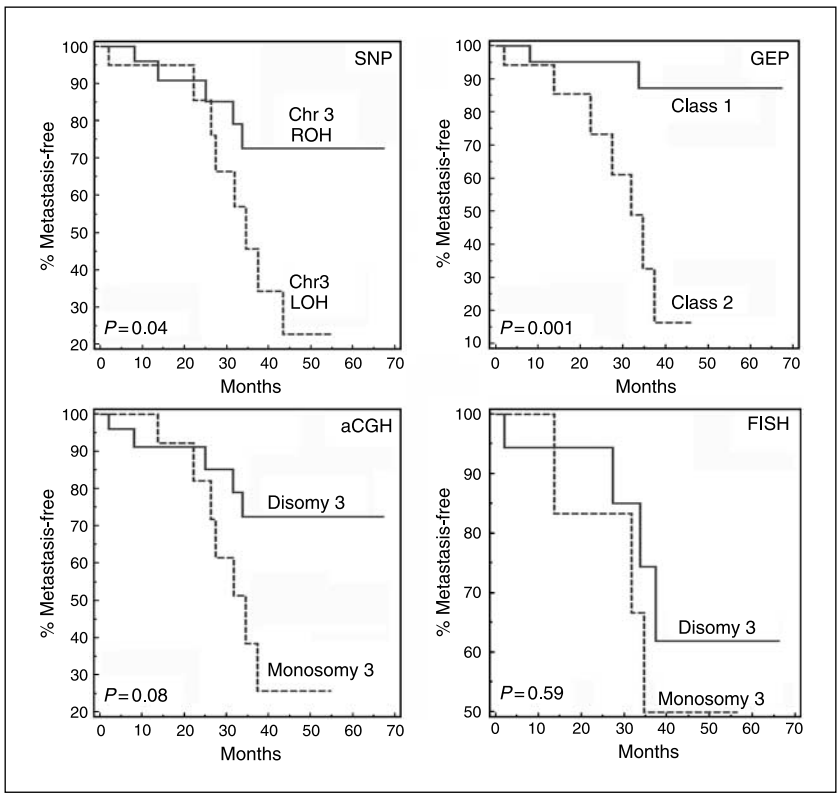

Fig. 2. Kaplan-Meier survival analysis for 53 tumors analyzed by the SNP, gene expression profiling (*GEP*), aCGH, and FISH.

to have sensitivity and specificity superior to other clinical, pathologic, and molecular features. Hence, patients were assigned to the 'metastasis-free' group if they had no evidence of metastasis with at least 5 years follow-up or if their tumor exhibited the class 1 expression signature. Patients were assigned to the 'metastasis' group if they had developed metastasis or if their tumor exhibited the class 2 signature. The SNP LOH assay was superior to both of the monosomy 3 detection methods, with sensitivity and specificity of 95.2% and 80.8%, respectively, positive and negative predictive values of 80.0% and 95.5%, respectively, and positive and negative likelihood ratios of 4.95 and 0.06, respectively.

Discussion

We have shown previously that our RNA-based classifier is a more accurate prognostic indicator than monosomy 3 or clinicopathologic factors in uveal melanoma (13, 18). Nevertheless, we recognized the need to develop a DNA-based assay both as a supplement to the RNA-based classifier and as an alternative when RNA of sufficient quality for expression profiling is not available. The DNA alteration most strongly associated with metastatic disease is monosomy 3 (4–12). In this study, we showed that a SNP-based assay for detecting LOH of chromosome 3 was superior to assessing monosomy 3 using chromosome counting techniques, such as FISH and aCGH. LOH could be inferred from SNP analysis even when matching normal DNA was not available, as shown previously for other cancers (19). Further, intratumoral genetic heterogeneity is a significant source of error when assessing monosomy 3 by FISH (20), whereas SNP was able to discern LOH in the presence of contaminating heterozygous cells.

An important advantage of SNP is its ability to detect isodisomy 3. Indeed, all three false-negative calls by aCGH were due to isodisomy. Importantly, isodisomy 3 carries the same prognostic significance as monosomy 3 but is not detected by FISH, CGH, and other techniques that count the number of chromosomes. The frequency of isodisomy 3 in our study was ~6% of all uveal melanomas and ~16% of class 2 tumors. Consequently, the false-negative rate associated with monosomy 3 detection methods could significantly compromise their predictive accuracy. Isodisomy 3 also has important biological implications. The fact that isodisomy 3 is associated with the high-risk class 2 expression signature and metastasis suggests

Table 2. Predictive accuracy of molecular prognostic tests

Prognostic factor	Sensitivity (%)	Specificity (%)	Positive likelihood ratio	Negative likelihood ratio	Positive predictive value (%)	Negative predictive value (%)
LOH (SNP)	95.2	80.8	4.95	0.06	80.0	95.5
Monosomy 3 (FISH)	77.8	64.7	2.20	0.34	53.8	84.6
Monosomy 3 (aCGH)	85.0	72.0	3.04	0.21	70.8	85.7

that loss of chromosome 3 is not only a marker of metastasis, and that its pathologic effect is not simply due to haploinsufficiency. Rather, this observation implies that the copy of chromosome 3 that is retained in monosomy 3 tumors is defective at one or more tumor suppressor loci, and that it is this ostensibly defective copy that is duplicated in isodisomy 3 tumors.

We used as the basis for our LOH assay the recently developed technique of SNP-mass spectrometry, which allows rapid, inexpensive, and high-throughput analysis of many SNPs (16). This technique benefits from the rapidly increasing database of SNPs and their frequencies across the genome, and it is likely to become more efficient as additional SNPs are identified and incorporated into high-density array formats. For this initial proof-of-principal study, a relatively small number of SNPs were used, and only high confidence calls were included. Because loss of chromosome 3 that is associated with metastasis almost always involves the entire chromosome, rather than small interstitial deletions (21), we were able to achieve accurate results with relatively few SNPs. Nevertheless, we plan to interrogate more SNPs to determine whether greater density of SNP coverage would increase the statistical power of the assay. Microsatellite analysis is another approach for detecting LOH that has been used in uveal melanoma (22, 23). However, the scalability and low cost of the SNP-mass spectrometry technology will likely lead to its increased use in lieu of microsatellite analysis (15). As a greater density of SNPs is incorporated into the testing platform, the increased saturation of chromosome 3 will further increase testing accuracy.

The superior predictive accuracy of the SNP platform may be highly relevant because this test could be used in the future to determine whether a patient will receive adjuvant systemic therapy. Because this was a relatively small study, further validation of the SNP platform is under way in a larger, prospective study.

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