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Genomic microarray analysis on formalin-fixed paraffin-embedded material for uveal melanoma prognostication

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Cytogenetic alterations are strong outcome prognosticators in uveal melanoma (UVM). Monosomy 3 (-3) and *MYC* amplification at 8q24 are commonly tested by fluorescence in situ hybridization (FISH). Alternatively, microarray analysis provides whole genome data, detecting partial chromosome loss, loss of heterozygosity (LOH), or abnormalities unrepresented by FISH probes. Nonfixed frozen tissue is conventionally used for microarray analysis but may not always be available. We assessed the feasibility of genomic microarray analysis for high resolution interrogation of UVM using formalin-fixed paraffin-embedded tissue (FFPET) as an alternative to frozen tissue (FZT). Enucleations from 44 patients (clinical trial NCT00952939) yielded sufficient DNA from FFPET (n = 34) and/or frozen tissue (n = 41) for comparative genomic hybridization and select single nucleotide polymorphism analysis (CGH/SNP) on Roche-NimbleGen OncoChip arrays. CEP3 FISH analysis was performed on matched cytology ThinPrep material. CGH/SNP analysis was successful in 30 of 34 FFPET and 41 of 41 FZT samples. Of 27 paired FFPET/FZT samples, 26 (96.3%) were concordant for at least four of six major recurrent abnormalities (-3, +8q, -1p, +6p, -6q, -8p), and 25 of 27 (92.6%) were concordant for -3. Results of CGH/SNP were concordant with the CEP3 FISH results in 27 of 30 (90%) FFPET and 38 of 41 (92.6%) FZT cases; partial -3q was detected in two CEP3 FISH–negative cases and whole chromosome 3, 4, and 6 SNP-LOH in one case. CGH detection of -3, +8q, -8p on FFPET and FZT showed significant correlation with the clinical outcome measures (metastasis development, time to progression, survival). Results of the UVM genotyping by CGH/SNP on FFPET are highly concordant with those of the FZT analysis and with those of the CEP3 FISH analysis, and therefore CGH/SNP is a practical method for UVM prognostication. Genome-wide coverage provides additional data with potential relevance to UVM biology, diagnosis, and prognosis.

Keywords Uveal melanoma, microarray, genomic, FFPE, prognostic

Uveal melanoma (UVM) is a malignant neoplasm of neural crest–derived melanocytes in the uveal tract and is the most common primary ocular malignancy in white adults (mean age-adjusted incidence in the United States of 5.1 cases per million population) (1). In addition to race, ocular melanocytosis is a well-established predisposing factor for UVM (2). Overall, UVM has a 50% risk of metastasis within 15 years (3), with the liver as the most common site (4), and a mortality rate of 40–50% (3,5). Despite advances in detection

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and early diagnosis, the prognosis of UVM has remained essentially unchanged for decades (1).

Although histologically similar to skin melanoma, UVM has unique genetic abnormalities, some of which are shared by cutaneous dermal melanocytic lesions, such as blue nevi. Several clinicopathologic characteristics, such as age, size, extension, cell type (epithelioid vs. spindled), mitoses, necrosis, extracellular matrix loops, and tumor-infiltrating lymphocytes, have been correlated with outcome in UVM. However, assessment of many of these features is subject to substantial interobserver variability and lack of standardized criteria. Advances in the molecular biology of UVM identified specific genetic alterations with excellent prognostic value. Chromosome 3 monosomy (-3), amplifications involving the q arm of chromosome 8 (+8q) harboring the *MYC* oncogene, and other molecular cytogenetic alterations (-1p, +6p, -6q, -8p) have been recognized as strong predictors of metastases and death in UVM (6–15).

Monosomy of chromosome 3, commonly assessed by fluorescence in situ hybridization (FISH), has consistently been associated with metastases and adverse clinical outcome (6–9,16). Losses at 1p, 6q, and 8p and gains at chromosome 6p and 8q have also been documented in patients with UVM metastases, using FISH, multiplex ligation-dependent probe amplification (MLPA), and array-based comparative genomic hybridization (CGH) analyses. Chromosome 3 partial loss (17–19) or copy neutral loss of heterozygosity (LOH) (20), undetectable by chromosome enumeration probes, have been detected in a subset of UVM by single nucleotide polymorphism (SNP) arrays and MLPA. Several distinct RNA signatures associated with metastasis in UVM have also been identified using expression arrays (21) and make the bases of a commercially available gene expression profiling assay for UVM prognostication (22).

Analysis by CGH/SNP microarray, generating high resolution multichromosome data, has emerged as an alternative to the traditionally limited FISH panel testing for UVM prognostication. Whereas DNA from frozen, nonfixed tumor tissue (FZT) is considered of high enough quality for usage in CGH analysis, procurement of sufficient FZT is often challenging and not always possible. Recently, improved methods of DNA extraction from formalin-fixed paraffin-embedded tissue (FFPET) have enabled the use of common histologic tissue material for genomic analysis with good results (23).

The objective of this study is to demonstrate the validity of CGH/SNP microarray analysis on DNA extracted from FFPET samples as an alternative to FZT material, for clinical outcome prognostication in a consecutive series of UVM enucleations. Array analysis of DNA extracted from matched FZT served as the reference data set for validation.

Materials and methods

Specimens

The study included samples from 44 patients with UVM on clinical trial NCT00952939 at our institution (Cleveland Clinic). Specimens consisted of fine needle aspiration (FNA) material and matched enucleation-derived FFPET and/or FZT samples.

CGH/SNP

DNA for CGH/SNP analysis was extracted with a proteinase K digestion and phenol protocol from FFPET (23) samples and using the Gentra Puregene Blood Kit (Qiagen, Venlo, The Netherlands) for FZT samples. DNA of sufficient quantity and quality for CGH/SNP was obtained from 34 FFPET and 41 FZT representing the 44 patients. DNA isolated from the specimens was fluorescently labeled with cyanine dye Cy5 and control DNA labeled with cyanine dye Cy3 using the NimbleGen Dual-Color DNA Labeling Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Labeled products were cohybridized to the NimbleGen OncoChip v1.2 or OncoChip v2 arrays (Roche) with oligonucleotide coverage biased to more than 1,800 cancer features. The arrays feature a background whole genome resolution of approximately 140 kb that is increased to approximately 10 kb in the regions of interest. OncoChip v2 arrays also feature backbone SNP coverage, affording combined CGH/SNP results.

FISH

FISH with a probe for the CEP3 region was performed on FNA-derived ThinPrep slides (Hologic, Bedford, MA) and interpreted as previously described (24).

Data analysis

Arrays were scanned at 5 μ with a NimbleGen MS 200 Microarray Scanner (Roche) and images were processed with MS 200 1.0 Scanning Software (Roche), Signature Genomics NG Packager 1.0 (PerkinElmer, Waltham, MA), and NimbleScan 2.6 (Roche). Microarray data was analyzed using Signature Genomics Oncoglyphix software (PerkinElmer). Abnormalities were automatically noted by the software when $\log_2$ ratio shifts were equal to or exceeded 0.3, 0.2, or 0.1 and the number of consecutive oligonucleotides in the abnormality was 5 or more, 100 or more, or 1,000 or more, respectively. A standard deviation of all nonshifted $\log_2$ ratio values was also calculated for each sample as a metric of array data quality. Additional abnormalities were manually noted when detected via visual inspection of the experimental data points for each chromosome. Each notation was classified regarding copy change (two-copy loss through four-copy gain), abnormality type (terminal, interstitial, or other), and level of significance (benign, unclear, or significant).

Calculations of clinical parameters (sensitivity, specificity, positive and negative prediction value, and 95% confidence interval) for FFPET-CGH analysis compared with FZT-CGH analysis as the gold standard, and FFPET/FZT-CGH analysis compared with FISH analysis as the gold standard, as well as Fisher exact test calculations and Kaplan–Meier curves with log-rank analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

Comparison of CGH/SNP analysis on FFPET vs. FZT

CGH analysis was technically successful in 30 of 34 FFPET and 41 of 41 FZT samples from UVM enucleations (Table 1),