Transvitreal Retinochoroidal Biopsy Provides a Representative Sample From Choroidal Melanoma for Detection of Chromosome 3 Aberrations

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Choroidal and ciliary body melanoma is the most frequent intraocular primary tumor in adults. It results in metastatic spread predominantly to the liver in 50% of cases and there is currently no effective treatment for disseminated disease.1 Accurate prognostication of choroidal and ciliary body melanoma patients has great implications for both clinical management and follow up.2

Identification of genetic alterations has provided a more accurate prognosticatio of patients compared with histological and clinical factors alone.2,3 Loss of chromosome 3 and gain of chromosome 8q are significantly associated with poor survival and are widely used as prognostic markers.4-6 In addition, any abnormal copy number of chromosomes 3 and 8 has recently been shown to be associated with a poor outcome.3 New discoveries of genetic mutations in uveal melanoma-related genes including BAP1, GNAQ, GNA11, SF3B1 and EIF1AX have led to further prognostic stratification.9 A classification system based on gene expression profile (GEP) divides patients into class 1 with a good prognosis and class 2 with a high risk of developing metastatic disease.10,11 Progress in treatment of the primary tumor has led to the possibility of eye-saving treatment such as radiotherapy in approximately two-thirds of cases.12 This necessitates a biopsy in order to obtain a tissue sample for genetic testing. Genetic heterogeneity in choroidal and ciliary body melanoma has previously been demonstrated for chromosome 3 by FISH and for chromosomes 1p, 3, 6 and 8 by multiplex ligation-dependent probe amplification (MLPA).13-16 A recent study also demonstrated discordant results in terms of GEP between two fine needle aspiration biopsies from the same tumor due to genetic heterogeneity.17 This has led to the conclusion that a single random tissue sample might be insufficient for prognostic testing.13 Thus, there could be a risk of genetic misclassification and consequently, wrong clinical decision making based on a biopsy sampled from a single tumor site. The transvitreal retinochoroidal (TVRC) biopsy obtains a larger tissue sample...
The diagnosis of choroidal and ciliary body melanoma was histologically verified in all cases. Nine tumors were too small to allow for the microdissection of at least three individual hexagonal tumor samples and were therefore excluded. Thus, 27 patients were finally included in the study. Genetic information from MLPA analysis of the biopsy was available in eight of the included patients and fresh frozen tissue from the TVRC biopsy was also available in 11 patients and was analyzed with MLPA. The procedures of routine FISH and MLPA analyses on tumor biopsies have been described previously.3 This retrospective study was approved by the local ethics committee in Copenhagen (jr. no. H-2-2013-064) and the Danish Data Protection agency (jr. no. 30-0120) and was conducted in accordance with the tenets of the Declaration of Helsinki. Oral informed consent was obtained prior to treatment in all patients.

**Biopsy Technique**

The surgical technique and potential intraocular complications of the minimally invasive TVRC biopsy (Constellation and 25 GA Total Plus; Alcon Laboratories, Inc., Fort Worth, TX, USA) has been described previously.12 In brief, the vitreous cutter was led through the vitreous cavity and retina without cutting, leaving the vitreous body intact. Activation of the vitrector was first initiated inside the tumor, using a low cut-rate, high suction (600 mm Hg) and a biased open duty cycle. Cutting was performed until tumor cells were observed in the vitrector tubing by the assistant. As the vitreous cutter continuously aspires tissue once inside the tumor, only the tumor size determines the sample size. In practice, an incomplete “mini en-doresection” is performed and multiple passages are therefore unnecessary.

**Genetic Analysis**

Archived FFPE tumor blocks from the 27 patients were collected and four 8-μm sections were cut from each block on a microtome. An additional 4-μm section was stained with H&E. Each section was manually microdissected according to a hexagonal grid of 3.5 mm as shown in Figure 1. A preceding pilot study showed that microdissected samples less than 3.5 mm did not provide reproducible amounts of DNA for MLPA analysis. Each microdissected sample was transferred to an Eppendorf tube and labeled with a coordinate from one to five according to horizontal (base-apex) location and from one to eight according to horizontal (anterior-posterior) location (Fig. 1). Controls consisted of FFPE tissue from nontumor eyes. We extracted DNA from the microdissected samples and controls using a purification kit according to the manufacturer’s instructions (Maxwell 16 FFPE Plus LEV DNA; Promega, Madison, WI, USA) and quantified on a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, Wilmington, DE, USA). We performed MLPA using a uveal melanoma probe mix (SALSA MLPA P027; MRC-Holland, Amsterdam, The Netherlands) with the addition of approximately 0.5% bovine serum albumin (BSA; protocol provided by the Liverpool Ocular Oncology Research Group). Data generated by subsequent capillary electrophoresis on a genetic analyzer (model 3130x; Life Technologies, Carlsbad, CA, USA) was analyzed using the Coffalyzer.Net data analysis software from MRC Holland (v.140721.1958). The interpretation of all MLPA results in the study was performed by one specialist (MTA) who was blinded to the genetic results from the matched biopsy and the geographic location of the samples within the tumor. Chromosomal aberrations were recorded as either significant (if the majority of probe signals differed significantly from the

**METHODS**

All patients with choroidal or ciliary body melanoma treated by the Ocular Tumor Division at the Copenhagen University Hospital between January 1, 2009 and December 31, 2013, were reviewed for this retrospective study. A total of 39 patients had a tumor with a height of at least 5 mm and had had a minimally invasive TVRC biopsy performed to cytologically confirm the diagnosis before the eye was enucleated and subsequently tested for genetic aberrations.12 None of the patients had received radiotherapy prior to enucleation. Information on aberrations of chromosome 1p, 3, 6 and 8 from routine FISH analysis on the biopsy specimen was available in 37 of the patients. The formalin-fixed paraffin-embedded (FFPE) tumor block from one patient was not available. Consequently, hematoxylin and eosin (H&E) sections from the remaining 36 tumors were reviewed and a grid of 3.5-mm hexagons was applied as shown in Figure 1A. The purpose of this study was to evaluate whether genetic testing with minimally invasive 25-gauge TVRC biopsy can be used as a genetic prognostic tool in the management of choroidal and ciliary body melanoma. Furthermore, the aim was to investigate the clinical implications of genetic intra-tumor heterogeneity.

**FIGURE 1.** Sections from matched choroidal and ciliary body melanomas served as a model for genetic heterogeneity and as controls for biopsy results. (A) A grid of 3.5-mm hexagons was applied to each tumor and labeled with a coordinate according to vertical (base-apex) location and horizontal (anterior-posterior) location. (B-D) Shows the gradual microdissection of an 8-μm tumor section.
reference samples) or equivocal (if the majority of probe signals showed a trend toward loss or gain, but only a small subset of probes differed significantly from the reference samples).

**Statistical Analysis**

Descriptive statistics were reported as mean and standard deviation when normally distributed and as median, range (R), and interquartile range (IQR) when the data was skewed. Chromosomal aberrations of chromosomes 3 and 8 were analyzed with MLPA and FISH. The ability of the biopsy to detect the same genetic aberrations as identified by MLPA of the FFPE tumor section was compared with the biopsy test results. The heterogeneity of the individual chromosomes is summarized in Table 2. Chromosome 3 only showed heterogeneity in three tumors (13%), whereas chromosome 8 showed heterogeneity by MLPA in 13 tumors (46%). A total of 13 tumors demonstrated heterogeneity of chromosome 3 or 8. Examples of homogeneous and heterogeneous tumors are shown in Figure 2. There was no significant difference in tumor size between the 13 heterogeneous and 11 homogeneous uveal melanomas with respect to AJCC tumor size (P = 0.18) as shown in Figure 3. The probability of finding chromosome 3 or 8 abnormalities increased (odds ratio [OR] = 1.65, P = 0.049) toward the base of the tumor. This tendency was also found when chromosome 8 was evaluated individually, although it was not significant (OR = 1.50, P = 0.12).

**Results**

A total of 200 specimens from 27 enucleated eyes were analyzed. The quality of the extracted DNA from the FFPE tissue was in three cases not suitable for MLPA. Consequently, a total of 177 specimens from 24 tumors were available for analysis. The median number of tumor samples dissected from each tumor was 7 (R: 3–18; IQR: 6–8). Baseline characteristics are summarized in Table 1.

**Genetic Heterogeneity**

MLPA identified chromosomal heterogeneity in 20 of 24 tumors when only significant MLPA results were regarded as abnormal and equivocal MLPA results were regarded as normal. Heterogeneity of the individual chromosomes is summarized in Table 2. Chromosome 3 only showed heterogeneity in three tumors (13%), whereas chromosome 8 showed heterogeneity by MLPA in 11 tumors (46%). A total of 13 tumors demonstrated heterogeneity of chromosome 3 or 8. Examples of homogeneous and heterogeneous tumors are shown in Figure 2. There was no significant difference in tumor size between the 13 heterogeneous and 11 homogeneous uveal melanomas with respect to AJCC tumor size (P = 0.18) as shown in Figure 3. The probability of finding chromosome 3 or 8 abnormalities increased (odds ratio [OR] = 1.65, P = 0.049) toward the base of the tumor. This tendency was also found when chromosome 8 was evaluated individually, although it was not significant (OR = 1.50, P = 0.12).

**Validity of the TVRC Biopsy**

In 16 cases, both MLPA and FISH of the TVRC biopsy were available, and in an additional eight cases, only FISH was available. The TVRC biopsy identified the tumor clone with the most chromosomal aberrations in 44% of cases analyzed with MLPA, whereas FISH only detected 25% of the cases.
The TVRC biopsy discriminated between normal and abnormal genetic status of chromosomes 3 or 8 in all cases with MLPA (sensitivity 100%), while FISH analysis detected 19 of 21 abnormal tumors (sensitivity 90%). Two-by-two (2 × 2) tables of paired tumor and biopsy results for chromosome 3 and 8 are shown in Table 3. Analysis with MLPA produced a higher detection rate of chromosomes 3 (100% vs. 89%) and 8 (75% vs. 68%) compared with FISH analysis (Table 5). However, analysis of the biopsies identified an additional three cases with more than two copies of chromosome 3 (patient IDs 8, 9, and 20 in Fig. 4). These chromosome 3 aberrations were missed by MLPA both in the biopsy specimen and in the whole tumor section. As neither MLPA nor FISH proved to be capable of detecting all chromosomal aberrations, the specificity of the TVRC biopsy could not be calculated as the true genetic status of the tumors was not known.

Any aberration of chromosome 3 or 8 was identified in all 21 cases (sensitivity = 100%) when MLPA and FISH data were combined. Furthermore, the sensitivity for chromosomes 3 and 8 was 95% and 79%, respectively, when all 24 biopsies were considered with a combination of FISH and MLPA data.

### Tissue Yield by the 25-Gauge TVRC Biopsy

The vitrector-based biopsy yields a large amount of tissue compared with FNAB. Figure 5 compares the maximal theoretical tissue yield of a 25 gauge vitrector-based system and a single-pass FNAB. The theoretical volume of FNAB is based on tumor height and the inner diameter of the 25- (0.26 mm), 27- (0.21 mm), and 30-gauge (0.159 mm) needles while the volume of the TVRC biopsy is based on the inner diameters of the cutter shaft (0.297 mm) and the tubing (0.5 mm) combined with the lengths of the Alcon 25-gauge vitrector system as shown in the lower panel in Figure 5.

### DISCUSSION

Despite the high degree of heterogeneity of the melanomas investigated, the minimally invasive 25-gauge TVRC biopsy identified all patients with a high risk of developing metastatic disease when a combination of FISH and MLPA was used as chromosomal tests. High-risk patients were defined as patients with aberration in either chromosome 3 and/or chromosome 8-3.

With regard to the determination of chromosome 3 status, the sensitivity of the TVRC biopsy was 89% (n = 24) with FISH analysis and 100% in the subgroup of 16 biopsies with available MLPA results (Table 3). Interestingly, in three of the 24 tumors, FISH analysis of the biopsy identified genetic alterations of chromosome 3, which were not shown by MLPA analysis in the corresponding FFPE tumor section or MLPA analysis of the biopsy. In these three cases, FISH analysis showed more than three copies of chromosome 3 and in two cases, more than three copies of chromosomes 6 and 8 as well. It has previously been shown that MLPA and FISH results diverge in highly genetically unstable tumors with polysomic chromosomes. MLPA does not detect polyploidy of the entire genome, thus FISH analysis is a helpful supplement to identify those cases that could otherwise lead to misinterpretation of MLPA results as shown in patient 9 (Fig. 4).

Genetic heterogeneity of chromosome 3 was only identified in 3 of 24 tumors (13%). This is consistent with the findings of Dopiera et al. They demonstrated genetic heterogeneity in MLPA probes for chromosome 3 in 50% of cases; however, the heterogeneity of the individual probes only led to contradictory interpretation of intratumor MLPA results for chromosome 3 in 4 of 32 uveal melanomas (12.5%). The detection of chromosome 3 heterogeneity varies in the literature: one study demonstrated multiple clones with different percentages of monosomy 3 with FISH, but no focal heterogeneity with regard to chromosome 3 status. Meier et al. found no discrepancy for chromosome 3 status when they compared two samples obtained from different locations in the same tumor in a total of 43 uveal melanomas. Other studies found heterogeneity of chromosome 3 in 5 of 22 cases (23%), 7 of 50 cases (14%), 14 of 22 cases (32%), and 3 of 17 cases (18%). It was recently demonstrated that discordant GEP class was seen in 9 of 80 cases (11%) when two random FNAB biopsy samples from the same tumor were compared. Thus, even though heterogeneity of uveal melanoma seems to be limited in most cases it still entails the risk of genetic misclassification, and FNAB has been criticized for not obtaining a truly representative sample. The TVRC biopsy has the potential to harvest a substantially larger proportion of the tumor tissue compared with FNAB (Fig. 5) and should therefore be more representative of the genetic changes in the tumor. However, an aberration of chromosome 3 was still missed in one case (ID 15, Fig. 4). Furthermore, one case of melanoma-related death was observed among patients with a normal TVRC biopsy of chromosomes 3 and 8 in our previously described consecutive cohort of 153 patients. This patient was included in the present study, but was unfortunately excluded from the final analysis due to poor DNA quality of the FFPE tumor tissue. The TVRC biopsies from both cases of genetic misclassification were only tested by centromeric FISH probes. This technique does not benefit from a large sample size as only 200 cells are evaluated. In addition the centromeric probe does not identify chromosomal changes away from the centromere, thus it is likely that MLPA analysis of the biopsy specimen would have...
identified partial abnormalities of chromosome 3. We therefore suspect that the two cases of genetic misclassification could be due to analysis technique rather than sampling procedure.

There was no association between large tumor size and genetic heterogeneity ($P = 0.65$), which was consistent with the study by Dopierala et al. Genetic analysis showed an association between basal location of the microdissected sample and abnormal copy number of both chromosomes 3 and 8 ($P = 0.049$). This finding has previously been suggested by Schoenfield et al. who identified 3 of 17 tumors with heterogeneity of chromosome 3 that all demonstrated monosomy 3 at the base and disomy 3 at the apex. However, another study failed to show the same association, thus this finding needs to be tested in a larger study population.

All biopsies in the study were obtained in vivo as part of the primary enucleation, thus the results of the biopsies reflect the authentic clinical setting. Additionally, the eye was removed in the same setting in all cases without receiving any kind of radiation and there was no delay between biopsy and enucleation. In 16 patients, MLPA biopsy results could be compared to MLPA results from the FFPE tumor section. MLPA of the biopsy identified chromosome 3 in all cases while aberrations of chromosome 8 were missed in three cases. This could be due to the more frequent heterogeneity of chromosome 8 compared with chromosome 3 (Table 2) or because chromosome 3 specific probes is more abundant ($n = 19$) in the MLPA kit than the chromosome 8 specific probes ($n = 6$).

The study population consisted only of large melanomas (median tumor height: 9 mm) due to study design. This selection bias resulted in a high frequency of biopsies with chromosome 3 abnormalities (13/16 using MLPA and 20/24 using FISH), which has been previously demonstrated for large uveal melanomas. It would also be interesting to compare genetic status of small choroidal and ciliary body melanomas to biopsy results and we have previously shown that the 25-gauge TVRC biopsy obtains a sufficient sample for histopathological and genetic testing regardless of tumor size. However, it was not possible to assess the heterogeneity in smaller tumors using the present study method because smaller dissection samples would not have yielded enough DNA to perform the MLPA analysis. It could also be speculated that the risk of genetic misclassification with a biopsy would be higher in larger tumors, and a nonsignificant trend between tumor thickness and discordance between GEP results has previously been shown. However, this association could not be evaluated in the present study. This study did not describe the heterogeneity of choroidal and ciliary body melanoma at the cellular level. However, the microdissected samples corresponded to the amount of material obtained by a biopsy and were therefore relevant to examine with regard to the
clinical aim of this study. In eight cases, there was no MLPA result from the biopsy; thus the comparison of biopsy-obtained genetic profile relied on FISH while the analysis of the FFPE tumor section was performed by MLPA analysis. This made it difficult to evaluate the isolated validity of the TVRC biopsy in these eight cases. An aberration of chromosome 3 was missed in one of these eight cases but the FISH result of the biopsy did detect gain of chromosome 8 (patient ID 15, Fig. 4). Thus this

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MLPA sensitivity, 100%

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MLPA sensitivity, 75%

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MLPA sensitivity, 100%

| Biopsy specimens were analyzed using two methods: MLPA in 16 patients and FISH in all 24 patients. Formalin-fixed paraffin-embedded eye sections were analyzed with MLPA only and served as controls. Equivocal MLPA results were regarded as normal in this analysis.

FIGURE 4. Heterogeneity in the tumor detected by MLPA compared with biopsy results from MLPA and FISH analysis. Each study patient is represented by an ID number. The chromosomes are listed from top to bottom as 1p, 3p, 3q, 6p, 6q, 8p and 8q for each patient ID. The copy number of each tested chromosome is shown using colors. The heterogeneity of the whole tumor section is shown as a percentage. (G), equivocal gain; G, gain; (L), equivocal loss; L, loss; NA, not available; N, normal; P, polysomy.
patient was still assessed as “high risk” based on the genetic result from the biopsy. The quality of the MLPA data obtained from the FFPE tumor sections was inferior to that routinely obtained from fresh tissue. The results of this study should hence be interpreted with caution and ideally be confirmed in studies using fresh tumor material only. The concordance of MLPA results from archived FFPE tissue and snap frozen tissue does, however, seem to be acceptable.

In conclusion, this study demonstrates that the presence of heterogeneity of chromosome 3 in choroidal and ciliary body melanomas is limited while genetic heterogeneity of chromosome 8 is more frequent. The genetic heterogeneity does not seem to be associated with tumor size. Our results indicate that aberrations of chromosome 3 and 8 are more frequent in the base of the tumor; however, this finding needs to be reproduced in a larger study population. A correct match for all four tested chromosomes between MLPA of the biopsy and MLPA of the whole tumor section was only achieved in 43.8% of cases. However, the TVRC biopsy proved sufficient for differentiating between low risk and high risk patients with large uveal melanomas.

MLPA was superior to FISH in identifying partial chromosomal deletions, but chromosome 3 aberrations in tumors with great genetic instability and polyploidy of all chromosomes were only identified by FISH.

To secure valid prognostication of patients with uveal melanoma, we recommend either TVRC biopsy or multiple FNAB passes to obtain a sufficient sample size and avoid genetic misclassification due to tumor heterogeneity.

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**References**


