Comparison of Gene Expression Profiling and Chromosome 3 Analysis by Fluorescent in situ Hybridization and Multiplex Ligation Probe Amplification in Fine-Needle Aspiration Biopsy Specimens of Uveal Melanoma

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Keywords

Uveal melanoma · Choroidal melanoma · Molecular prognostication · Fine-needle aspiration biopsy · Fluorescent in situ hybridization · Gene expression profiling · Multiplex ligation probe amplification · Monosomy 3

Abstract

Purpose: The aim of this paper was to assess the concordance between results of DecisionDx-UM specific gene expression profiling (GEP) and fluorescence in situ hybridization (FISH) for chromosome 3 analysis, and between DecisionDx-UM GEP and multiplex ligation probe amplification (MLPA) in uveal melanoma undergoing intraoperative fine-needle aspiration biopsy (FNAB) for metastatic prognostication during brachytherapy. **Methods:** We retrospectively reviewed consecutive patients diagnosed with posterior uveal melanoma who underwent intraoperative FNAB prior to placement of an iodine-125 radioactive plaque between 2012 and 2014. Two cohorts of patients were identified: Cohort 1 – tumors in which both GEP and FISH results were ob-

tained, and Cohort 2 – tumors in which both GEP and MLPA results were obtained. **Results:** Forty-four patients were identified for Cohort 1. FISH and GEP results were discordant in 7 tumors (15.9%). Forty-three patients were identified for Cohort 2. MLPA and GEP were discordant in 7 tumors (16.3%). **Conclusions:** Discordance between GEP and chromosome 3 status by FISH and MLPA occurred in our series at a rate of 15.9 and 16.3%, respectively. Caution must be advised when counseling a patient with a good-prognosis GEP “Class 1” result that the uveal tumor may actually harbor monosomy 3, which is associated with a poor prognosis for metastasis in nearly 20% of the patients.

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Introduction

Molecular prognostic biopsy for metastatic risk in the management of patients with uveal melanoma is standard of care at most major ophthalmic oncology practices in North America [1–8]. The loss of 1 copy of chromosome 3 (monosomy 3 [M3]) in the tumor tissue has been deter-

mined to be the risk factor most strongly associated with metastatic death originally reported in mostly large uveal melanoma [9–11]. Today, there are several options for tumor tissue testing to determine metastatic risk, both DNA and RNA based. Among DNA-based tests, fluorescence in situ hybridization (FISH) testing for the centromere region of chromosome 3 was first reported at our center in samples obtained through intraoperative fine-needle aspiration biopsy (FNAB) [12]. Multiplex ligation probe amplification (MLPA) for uveal melanoma prognostic testing was reported in the United Kingdom, and this DNA-based technique, which samples foci on chromosomes 1, 3, 6, and 8 is now available as a commercial test in North America [13, 14]. A gene expression profile test based on RNA has been available as a commercial test, grouping patients into a Class 1 gene expression profile (good prognosis) and a Class 2 gene expression profile (poor prognosis) [15, 16]. Both DNA- and RNA-based tests are considered to be acceptable platforms for determining a patient's metastatic prognosis when combined with clinical risk factors.

There is the assumption that a good prognosis is conferred for the patient who has a disomy 3 or a Class 1 result with molecular prognostic testing, and that a poor prognosis is associated with a M3 or Class 2 result. With several methods available for determining molecular prognosis, we sought to better understand the results of FISH, gene expression profiling (GEP), and MLPA testing in the same patient. We sought to determine how similar the prognostic result of GEP testing was compared to FISH, and GEP testing was compared to MLPA.

Materials and Methods

From November 1, 2012, to December 31, 2014, all patients who had been diagnosed with uveal melanoma, treated with brachytherapy, and who underwent intraoperative FNAB (intraoperative 30-gauge transscleral or 27-gauge transvitreal) for molecular prognostication were identified, and the cases were retrospectively reviewed. Only posterior uveal melanoma patients with both GEP and FISH or MLPA molecular test results were included. Patients with iris melanomas were excluded. FNAB technique consisted of a transscleral approach immediately after the anterior tumor border was marked on the sclera using transpupillary transillumination. Multiple passes (numbering between 4 and 7) were taken using a separate 30-gauge needle per pass. The needle was connected to tubing and attached to a 10-mL syringe, allowing an assistant to create suction while the needle was in the tumor. The assistant collected samples for cytopathology, cytogenetics, GEP, MLPA, and tissue banking. If the assistant did not retrieve an adequate biopsy specimen, an additional pass was performed. Additional maneuvers such as creating a scleral flap, suturing, or cryotherapy were not performed.

Cytogenetic testing for M3 on biopsy samples had been performed by FISH at our center since December 2004 and continued until June 2014 when the more accurate MLPA test became commercially available. After November 1, 2014, our center initiated GEP testing with the DecisionDx-UM (Castle Biosciences, Phoenix, AZ, USA) test on biopsy samples, together with FISH testing for M3 in patients undergoing treatment for uveal melanoma. MLPA testing (Impact Genetics, Bowmanville, ON, Canada) became available and was offered to all patients from June 1, 2014, to December 31, 2014, together with GEP testing, but FISH testing was not offered during this time due to the known improved accuracy of identifying M3 by MLPA.

Therefore, we examined 2 cohorts of patients: Cohort 1 consisted of patients who underwent intraoperative biopsy for molecular prognosis during brachytherapy with GEP and FISH (November 1, 2012, to January 31, 2014); Cohort 2 consisted of patients who underwent intraoperative biopsy for molecular prognosis during brachytherapy with GEP and MLPA (June 1, 2014, to December 31, 2014). Patient demographics and tumor characteristics were reviewed. The results of biopsy specimens, analyzed by cytopathology, FISH for M3 and 6p gain, GEP analysis with the DecisionDx-UM assay, and MLPA with the Impact Genetics assay were reviewed. A patient was excluded from their cohort if one of the molecular tests failed to amplify. Patient and tumor characteristics and the molecular prognostic test result from tumor biopsies were recorded. 'Concordance' between molecular prognostic tests was defined as when both Class 1 (either Class 1A or Class 1B) and disomy 3 were recorded in the same patient, or when Class 2 and M3 were recorded in the same patient. 'Discordance' between tests was defined as when Class 1 (either Class 1A or Class 1B) was found with M3, or when Class 2 was found with disomy 3 in the same patient.

Microsoft Excel (Microsoft Inc., Redmond, WA, USA) was used to calculate descriptive statistics and also to perform a two-tailed t-test to evaluate significance of patient age, tumor height, and ciliary body involvement between concordant and discordant cases in both cohorts.

Results

A total of 153 consecutive intraocular tumors underwent brachytherapy with intraoperative biopsy, including 141 choroidal melanomas from November 1, 2012, to December 31, 2014. Two cohorts of patients were identified based on prognostic assay availability.

Cohort 1 – Patients with Results from GEP and FISH for M3

Cohort 1 consisted of 44 patients diagnosed and treated for uveal melanoma in which intraoperative biopsy was performed for molecular prognosis with both GEP and FISH for M3. There were no test failures in this cohort. Patient and tumor characteristics are detailed in Table 1. Concordance and discordance of biopsy results in this cohort of patients are depicted in Figure 1. The over-

Fig. 1. Concordance of gene expression profiling (GEP) and fluorescent in situ hybridization (FISH). M3, monosomy 3.

Fig. 2. Concordance of gene expression profiling (GEP) and multiplex ligation probe amplification (MLPA). M3, monosomy 3.

Table 1. Cohort 1: DecisionDx-UM GEP versus FISH M3

Choroidal melanoma, <i>N</i>	44
Mean patient age, years	63 (range 34–88)
Male	52% (<i>n</i> = 23)
Mean tumor height	5.28 mm (SD = 3.57)
Mean tumor basal diameter	12.56 mm (SD = 2.58)
Mean follow-up	22.2 months (range 15.9–29.9)

GEP, gene expression profiling; FISH, fluorescent in situ hybridization; M3, monosomy 3; SD, standard deviation.

Table 2. Cohort 2: Decision Dx-UM GEP versus MLPA (Impact Genetics, Bowmanville, ON, Canada)

Choroidal melanoma, <i>N</i>	43
Mean patient age, years	61 (range 29–80)
Male	47% (<i>n</i> = 20)
Mean tumor height	4.54 mm (SD = 2.85)
Mean tumor basal diameter	12.33 mm (SD = 3.42)
Mean follow-up	8.9 months (range 5.4–11.4)

GEP, gene expression profiling; MLPA, multiplex ligation probe amplification; SD, standard deviation.

all concordance of GEP and FISH for M3 was 84.1%. The overall discordance was 15.9%. Among Class 1 cases, FISH identified 19.3% (6 in 31) as having M3, and among Class 2 cases, FISH identified 7.7% (1 in 13) as having disomy 3. There was no statistically significant difference between discordant and concordant cases when comparing tumor height ($p = 0.79$), patient age ($p = 0.57$), and ciliary body involvement ($p = 0.40$).

Cohort 2 – Patients with Results from GEP and MLPA for M3

Cohort 2 consisted of 49 patients diagnosed and treated for uveal melanoma in whom intraoperative biopsy was performed for molecular prognosis using both GEP and MLPA for M3. Six patients were excluded due to test failures (3 MLPA samples, 3 GEP samples), resulting in a total of 43 patients in Cohort 2. Patient and tumor characteristics are detailed in Table 2. Concordance and discordance of biopsy results in this cohort of patients is depicted in Figure 2. The overall concordance of GEP and MLPA for M3 was 83.7%. The overall discordance was 16.3%. Among Class 1 cases, MLPA identified 19.3% (6 in 31) as having M3, and among Class 2 cases, MLPA identified 8.3% (1 in 12) as having disomy 3. There was no statistically significant difference between discordant and concordant cases when comparing tumor height ($p = 0.50$) or patient age ($p = 0.055$).

There were no cases of confirmed metastatic disease in either cohort during the limited follow-up period of this study (cohort 1 – mean 22.2 months, range 15.9–29.9; cohort 2 – mean 8.9 months, range 5.4–11.4).

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Discussion

We have demonstrated that there is an approximately 84% agreement rate in molecular prognosis between FISH testing and GEP, and between MLPA testing and

GEP. The highest rates of discordance were found among Class 1 cases in both FISH/GEP and MLPA/GEP at 19.3%. This essentially meant that when GEP was used in conjunction with MLPA or FISH, about 1 in 5 patients with Class 1 tumors were identified as M3. Tumor height was not significantly different between concordant and discordant groups in both cohorts.

Molecular testing for prognosis in patients with uveal melanoma has become standard of care in the management of primary tumors. Studies have demonstrated that patients want to know their prognosis with respect to the development of metastasis, whether or not there are effective treatments for metastatic disease [17]. It has been suggested that information and less uncertainty may empower patients and help them better address aspects of their cancer and how it affects their life. The two main methods of molecular testing involve determining M3 status of the tumor [9, 10, 14], one is a DNA-based approach, and the other makes use of a commercially available RNA-based GEP test that groups patients into Class 1 (low risk for metastasis) and Class 2 (high risk for metastasis) [15, 18]. The literature supports the use of both tests as good predictors of metastatic outcome [19].

However, we have shown in this paper that what constitutes 'good prognosis', that is a disomy 3 or a Class 1 test result, does not necessarily correlate when tumor biopsy samples are taken from the same patient. In fact, both FISH testing and MLPA testing to determine the status of chromosome 3 within the tumor have a 15 and 16% rate of disagreement or discordance, respectively, when compared to GEP testing, thereby identifying more patients with a poor prognosis than with GEP, with MLPA being more sensitive than FISH in determining chromosome 3 status, which we and others have reported previously. Our results are similar to a report by Onken et al. [20], where 260 patients with uveal melanoma in their series underwent both GEP testing and a single-nucleotide polymorphism test (developed by the authors) for determining M3 status. There was a similar discordance rate of 14.6% (38 patients) where prognostic biopsy resulted in a Class 1 result and a M3 result. Regarding those patients with Class 2 and disomy 3 results, there was a 6.2% discordance rate in their study, compared to our 3.8% with FISH and 3.1% with MLPA.

It is not entirely clear why a consistent percentage of at least 15% of the patients (19% when MLPA was used) with a Class 1 test result should also have a M3 test result in our series as well as in the larger series by Onken et al. [20]. Although the published literature supports a good prognosis in the short-term, it is well known that Class 1 patients may

indeed develop metastasis and that the percentage of metastasizing Class 1 melanomas increases with longer follow-up than initial reports indicated. Perhaps DNA testing is more sensitive at detecting high risk for metastasis patients. RNA and its inherent instability compared to DNA may be more prone to handling error, and perhaps this may be the source of an inherently inaccurate test in the setting of GEP. It has also been suggested that the size of the tumor must be considered when interpreting a Class 1 or a Class 2 test result [21]. However, we found that the average tumor size was not statistically different between concordant and discordant groups of patients in our series.

Tumor heterogeneity may possibly account for the discordant test results [1, 22–25]. Each biopsy sample was obtained by an independent needle pass. Therefore, a separate area of the tumor with potentially different molecular make-up may have been sampled. However, the consistency of the discordance rate of 15% (patients with Class 1 and M3) suggests that the Class1/M3 patients may be a different and unique subset whose test results may not be entirely attributable to random heterogeneity.

Although there are no long-term published reports on the rate of Class 1 uveal melanomas that ultimately metastasize, there are many anecdotal instances of patients initially diagnosed with a low-risk uveal melanoma that went on to develop liver metastasis. There are also instances of disomy 3 uveal melanoma that have metastasized. With MLPA and microsatellite techniques, and improved DNA-based testing over simple FISH testing, karyotype and CGH, cases of isodisomy 3 that can mistakenly give a good prognosis test with these methods, can now be detected resulting in a high risk for metastasis. Furthermore, the commercial MLPA test also sequences for a *GNAQ* or *GNA11* mutation in cases of disomy 3 to help confirm that actual uveal melanoma tissue has been submitted for analysis. GNA mutations have been reported in 85–91% of all uveal melanomas. With GEP testing, we and others have reported Class 1 and Class 2 test results when nonuveal melanoma tissue is sampled [26, 27]. Some degree of testing inaccuracy on the part of the commercially available gene expression profile test may also contribute to the more sensitive detection of M3 in DNA-based approaches.

It is important to keep in mind that metastatic prognosis is a multifactorial assessment [7]. One must exercise caution with a molecular test result alone. Using a multivariate analysis, Damato et al. [28] have established a robust database that makes use of not only chromosome 3 status but also patient and tumor clinical factors such as age, tumor size, and also other histological characteristics when available to determine prognosis.

Long-term studies are necessary to determine the true accuracy of available molecular testing platforms for prognosis, as metastatic disease becomes manifest in patients. At this point, however, there is sufficient data to use all prognostic testing methods (including clinical features alone) to help counsel patients about their cancer and systemic surveillance. Continued follow-up and evaluation of existing prognostic testing methods including future ones must be continuously studied.

Acknowledgments

This work was supported by the George E. and Ruth Moss Trust and an unrestricted grant from the Research to Prevent Blindness.

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Statement of Ethics

The study was performed in accordance with the United States Health Insurance Portability and Accountability Act (HIPAA) of 1996 and was approved by the Office of the Human Research Protection Program (Institutional Review Board) of the University of California, Los Angeles, CA, USA.

Disclosure Statement

Dr. Tara McCannel is an uncompensated member of the Advisory Board of Impact Genetics. All other authors report no relevant conflicts of interest.