Review

MOLECULAR PROGNOSTICS FOR UVEAL MELANOMA

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Purpose: To review laboratory methods, currently available commercial tests, caveats and clinical tips regarding prognostic analysis of uveal melanoma tissue.

Methods: A review of the literature was performed focused on the genetic abnormalities found in uveal melanoma cells, their correlation to the development of metastases, the validity of various laboratory approaches in their detection, and the existing commercially available tests for uveal melanoma prognostication.

Results: Numerous laboratory methods exist for analyzing genetic material obtained from uveal melanoma cells. Older tests have been gradually replaced with contemporary methods that are simpler with greater accuracy. Two commercially available assays exist which have not been directly compared—a gene expression profiling test has been validated directly through a large, prospective multicenter study and a DNA-based test which uses laboratory methods supported by extensive historical data.

Conclusion: There are myriad laboratory methods for prognostic analysis of uveal melanoma tissue. These tests were historically only available to those with access to an outfitted laboratory. Newer commercially available assays have increased the accessibility of prognostic biopsy for uveal melanoma. The various caveats that exist when considering and performing prognostic biopsy of uveal melanoma are discussed.

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Uveal melanoma eventually results in the mortality of approximately 40% to 50% of those it affects despite complete control of the primary tumor.[1–4] However, no evidence of metastatic disease is demonstrable at the time of uveal tumor diagnosis in the vast majority of patients.[5,6] Clinical metastases usually develop years after the diagnosis and treatment of their primary ocular tumor.[7] Eskelin et al estimate that undetectable circulating tumor cells, or "micro" metastasis, may have begun growing 2 to 3 years before the treatment of the ocular tumor in the patients who will go on to develop disseminated disease.[8]

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The potential of a particular patient's uveal melanoma to go on to cause metastasis is commonly referred to as the tumor "prognosis" although this is not technically accurate as the term prognosis is usually defined as the likely course of a medical condition.[9] In common use, the term may refer to the course of the ocular tumor/eye, the risk of the patient eventually developing metastasis, or the patient's risk of mortality, which are mutually exclusive. This review (and most work in this field thus far) focuses on the risk of development of metastases from a uveal melanoma.

Historically, the risk of metastases from a uveal melanoma has been predicted based on the study of the morphologic and pathologic features of the tumor. Tumor thickness, diameter, location, presence of extraocular extension, and histopathology have been consistently shown to independently predict the likelihood of developing metastatic disease.[4,10–13] More recently, studies of the genetic and molecular makeup of uveal melanoma through ocular tumor biopsy have

proven even more reliable (as a single modality) in estimating the prognosis of patients with uveal melanoma and may often be performed with a fine-needle aspiration biopsy or through analysis of formalin-fixed paraffin-embedded tissue.[14,15] However, recent data have suggested that considering uveal melanoma size may add predictive power to genetic analysis for estimating the likelihood of metastasis.[16,17]

Rationale for Estimating Risk of Metastasis

Limited data suggest that patients with uveal melanoma value information regarding their risk of developing metastases and estimated survival, even if it is unfavorable.[18,19] In addition, if it can be known which patients with uveal melanoma are most likely to go on to develop dissemination, theoretically treatments such as chemotherapy or radiation could be administered "prophylactically" in an effort to prevent or delay the development of clinically significant tumor spread. Patients at higher risk of metastases could also be monitored more closely for the development of radiologically detectable "macro" metastases, as detection of early manifestations might allow treatment initiation with a lower disease burden. Only minimally effective treatment exists for metastatic uveal melanoma, so some dismiss the potential benefits of the identification of these high-risk patients. Although this may have been more convincing in the past, limited emerging data suggest that systemic therapy may prolong survival in some patients[20,21] and clinical trials are enrolling based on the molecular signature of a patient's tumor,[22] potentially leaving untested patients behind. In addition, some centers tailor metastasis screening protocols based on the molecular phenotype of the uveal tumor.[23]

Methods for Genetic Analysis of Uveal Melanoma

The relationship between specific chromosomal aberrations in uveal melanoma tumor cells and the risk of patient development of metastasis has long been studied.[24] Several laboratory methods for detection of chromosomal abnormalities have been reported, and most remain relevant for clinical use today. Of note, there exist technical variations when performing the following tests that may vary significantly between individual laboratories and likely affect the sensitivity and specificity of their results. The newer technology of gene expression profiling (GEP) is also described below.

Traditional karyotyping was the first method used to detect genetic abnormalities in uveal melanoma cells in 1985.[25] The technique uses direct microscopy of stained chromosomes within cultured cells arrested during cell division and is capable of identifying only large genetic alterations, such as the duplication or loss of an entire chromosome or chromosome arm. Karyotyping provided the first evidence that uveal melanoma cells had a particular genetic signature such as Monosomy 3 and/or duplication or loss of part or all of Chromosome 8.[25–28] Since that time, more sensitive and cost-effective techniques have supplanted karyotyping for the study of uveal melanoma.

Fluorescence in situ hybridization (FISH) may be the most studied genetic method used for analysis of uveal melanoma. To detect chromosomal abnormalities, FISH uses oligonucleotide probes to target specific chromosomal targets in a specimen of DNA —hybridization occurs if the probe finds its target. The probes are combined with a fluorescent molecule that is then detected using microscopy when hybridization has occurred. The technique may identify small targets (such as single-nucleotide polymorphisms [SNPs]) or large targets (such as entire chromosome duplications) depending on the size of the probes used. Fluorescence in situ hybridization was first used to evaluate uveal melanoma in 1997 by Bercher,[29] and also by McNamara.[30] Although significant data have been published suggesting that FISH is an effective and accurate technique for genetic analysis in uveal melanoma cells, other data suggest significant variability in FISH results, perhaps in part secondary to subjectivity in analyzing the results.[31,32]

Comparative genomic hybridization (CGH) uses FISH technology to compare fluorescence between two different sources of DNA—such as that from uveal melanoma and normal cells. Like FISH, CGH is limited by the optical resolution of a microscope to detect fluorescence.[33] In terms of uveal melanoma, its use predates that of FISH and was first described in 1994, confirming that loss of Chromosome 3 and duplication of 8q were common in large tumors.[34] More recent data on CGH suggest that it has a 77% success rate in samples obtained by fine-needle aspiration biopsy (FNAB)[35] which is significantly lower than other contemporary techniques.

Multiplex ligation-dependent probe amplification (MLPA) was first used on archival uveal melanoma tissue in 2008[36] and also uses oligonucleotide probes to detect specific targets in a DNA sample. In contrast to FISH and CGH, however, hybridization is detected in MLPA through the use of the PCR. By amplifying the amount of target DNA in a sample, PCR permits much greater levels of probe hybridization than FISH or CGH. This allows for smaller probe targets and significantly increased sensitivity. This is particularly relevant for uveal melanoma, where smaller biopsy

samples are preferred (see "Important Caveats for Molecular Prognostication for Uveal Melanoma," below). In addition, MLPA is faster and less expensive to perform than FISH and likely has an increased sensitivity in detecting genetic alterations in uveal melanoma cells.[37] Alterations in Chromosome 3 and 8p in uveal melanoma cells detected by MLPA have been shown to be very effective at predicting the development of metastasis.[38,39]

Microsatellite analysis (MSA) is an alternative genetic analysis technique that combines the use of fluorescently labeled probes and PCR together to evaluate a sample when not enough tissue is available for standard MLPA.[40–42] Microsatellite analysis from FNAB samples has been shown to be highly accurate for detection of abnormalities in Chromosome 3,[41,43] but may be less accurate for identifying abnormalities in Chromosomes 6 and 8.[42]

Single-nucleotide polymorphism array and direct DNA sequencing analyses are automated and able to detect many different genetic abnormalities within a target sample of DNA. Single-nucleotide polymorphism detection has been shown to be comparable[44] or better[45] than FISH for detecting chromosomal abnormalities in uveal melanoma. However, these technologies are considerably more expensive than the aforementioned methods and are less often used in uveal melanoma.[31] Advanced "second-generation" DNA sequencing technology is currently under investigation for uveal melanoma.

Gene expression profiling of uveal melanoma is a novel technique that evaluates RNA (or "gene expression") instead of DNA. The method measures the amount and type of RNA in a sample using a gene chip to estimate the activity of a complement or profile of several genes.[46] Initial data regarding this technique suggested that it might be superior to more traditional methods for detection of genetic alterations,[45–47] and more recent data from a multicenter prospective study have validated that GEP performed on a sample obtained by fine-needle aspiration outperformed the presence or absence of monosomy of Chromosome 3 (as detected by an SNP assay) in the prediction of metastasis from uveal melanoma.[14]

Genetic Signature of Uveal Melanoma

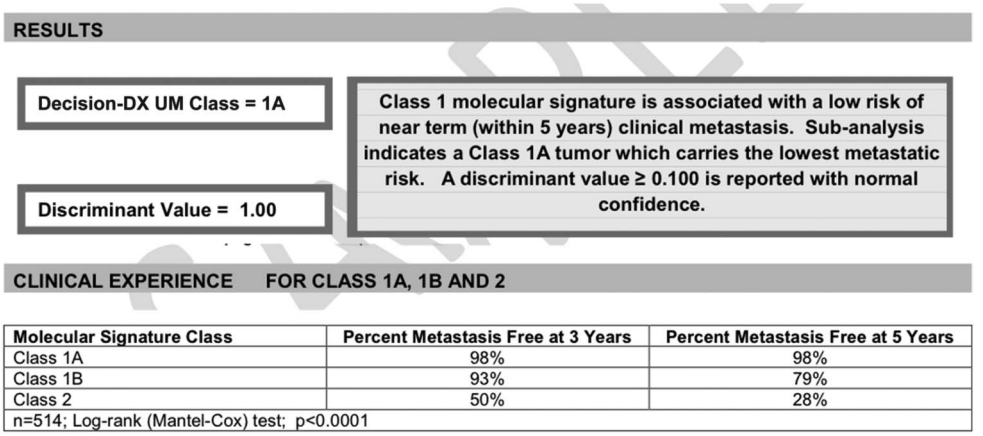
The aforementioned chromosomal analysis technologies have consistently found Chromosome 3 loss and 8q gains in uveal melanoma cells to highly correlate with the development of metastases in patients.[36–40,45,48] Losses of Chromosome 1, 8p, and 6q also seem to correlate with the development of metastasis[15,40,49–51] although comparatively less data for these associations exist. In contrast, gains in 6p seem to independently correlate with a reduced rate of development of metastasis.[38,40,52] Not surprisingly, these high-risk genetic abnormalities have been shown to cooccur with the histologic and morphologic features known previously to predict metastasis, such as increased tumor diameter, ciliary body involvement, and epithelioid cellularity.[38,48,49,53–55] Gene expression profiling analysis has suggested that certain genetic profiles (characterized by the increase and/or decrease in the expression of a certain subset of genes, many correlating to the chromosomal abnormalities listed above) may also predict the development of metastasis in uveal melanoma.[14,23,46,47] Significant data suggest that tissue obtained during fine-needle aspiration is usually sufficient for either chromosomal or GEP analysis.[15,32,35,43,56,57] Interestingly, although the genetic abnormalities characteristic of metastasizing uveal melanoma tumor cells can often be found in hepatic metastases from this disease, this relationship is not universal.[58]

Newly Discovered Genes With Prognostic Value in Uveal Melanoma

BAP1 mutations in uveal melanoma cells (as detected by one of several methods including MLPA, SNP analysis, microarray, etc.) seem to independently predict metastatic death and are associated with larger tumor size and ciliary body involvement.[59–62] Germline mutations in BAP1 also seem to be particularly common in familial uveal melanoma[63–67] and in familial cancer syndromes which commonly include cutaneous melanoma, mesothelioma, and other tumors.[68–72] BAP1 protein has tumor suppressor properties which remain incompletely understood.

PRAME expression in uveal melanoma cells also seems to be positively associated with larger tumor diameter, shorter time to metastasis, and increased risk of melanoma-associated mortality and is independently predictive when added to Class 1 or Class 2 distinction. PRAME is under investigation as a potential target for immunotherapy in cutaneous melanoma and may possibly constitute a treatment pathway for uveal melanoma in the future.[73,74]

GNAQ or GNA11 gene mutations are found in approximately 83% of uveal melanomas, but may also be found in nevi and do not seem to predict the development of metastases.[40,75,76] However, because these mutations are usually found in cells of melanocytic origin, detecting mutations in these genes has been proposed as a method to ensure tissue sampled during



Molecular Signature Class	Percent Metastasis Free at 3 Years	Percent Metastasis Free at 5 Years
Class 1A	98%	98%
Class 1B	93%	79%
Class 2	50%	28%
n=514; Log-rank (Mantel-Cox) test; p<0.0001		

Fig. 1. Excerpt from test result report from the Decision-Dx UM test from Castle Biosciences from a biopsy of uveal melanoma tissue.

biopsy is indeed melanocytic. This may be relevant, as the GEP test designed for uveal melanoma will provide a result without an error report even if the tissue sampled is not a melanoma at all.[77,78]

Mutation of the *EIF1AX* gene in uveal melanoma tissue (as opposed to wild-type) has recently been linked to a decreased risk of metastasis,[62,79] whereas tumors with a *SF3B1* mutation seem to show an increased risk of metastasis.[79]

Currently Commercially Available Systems for Genetic Analysis of Uveal Melanoma

Two commercially available systems exist for molecular prognostication of uveal melanoma and are both performed in Centers for Medicare and Medicaid Services Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories. The Decision-Dx UM test from Castle Biosciences Inc. has been available since 2009 and is based exclusively on the GEP test developed by Harbour et al that was validated in a prospective multicenter study.[14,23] The test evaluates the expression of 15 genes: 12 determined through experimentation to be the most powerful for mortality prediction and 3 "control" genes to account for the amount of genetic material in a sample.[80] In the aforementioned multicenter prospective study, Dr. Harbour's GEP test significantly outperformed Monosomy 3 (as detected by SNP analysis) in the prediction of metastasis.[14] Emerging data suggest that real-world use of the Decision-Dx UM test on FNAB samples provides a result in the vast majority of cases,[56,81] and the results seem to predict the development of metastases with a suitably high rate of accuracy in patients.[16,17,56]

Tissue for the Decision-Dx test is obtained through fine-needle tumor aspiration (through either direct *trans*-scleral or *trans*-retinal biopsy) that is placed into a buffer solution (provided by Castle Biosciences) and then shipped on dry ice to the commercial facility. The Decision-Dx UM test produces a result of Class 1, Class 1b, or Class 2 with a corresponding 5-year metastasis-free survival prediction of 98%, 79%, and 28%, respectively. A "discriminant value" is provided with the test result. This value correlates with the disparity between the molecular profile of the tumor and the established "hyperplane" differentiating Class results and is therefore a measure of reliability.[82] A discriminant value of < 0.01 is considered to correlate with an unreliable test result (Figure 1).

The uveal melanoma genetic test from Impact Genetics Inc. has been available since 2014 and uses a mix of MLPA and MSA to evaluate for complete or partial loss, duplications, or isodisomy of Chromosomes 1p, 3, 6, and 8 (Figure 2). In addition, genetic sequencing of the GNAQ and GNA11 genes is offered for select specimens. As mentioned earlier, the identification of these genes in a sample may provide supporting evidence that melanocytic cells (and therefore the correct target) were indeed biopsied. The test also takes into consideration the actuarial data based on the patient's age in determining their result (these data are also available online for free at ocularmelanomaonline.org) as well as tumor morphologic characteristics such as size, location, and cell type.[22,83] A purported theoretical advantage of the Impact test is that, because it uses MLPA and MSA, it may be performed on irradiated tissue, although this is not recommended in most patients.[84] Although the Impact test is based on traditional uveal melanoma genetic analysis technologies that have been evaluated for decades (MLPA and MSA), it has not been prospectively validated in patients like the Decision-Dx UM test has been. No head-to-head comparison of the Castle Biosciences Inc. and Impact Genetics Inc, tests has been performed.

Summary

The patient's tumor shows disomy 3. *GNAQ* sequencing confirms tumor was studied. When combined with the provided clinical and histomorphological data, multivariant analysis predicts the following:

Uveal Melanoma Survivorship Prediction[10, 11]

Survival	Year 3	Year 5	Year 10
Control*	97%	94%	85%
Patient	98%	77%	50%

*age and sex matched general population control group

Test results (actual test values attached)

- MLPA: Our lab tested a sample from the patient's uveal melanoma using the SALSA PO27.C1 Uveal Melanoma kit (MRC Holland) and determined that the patient's sample showed:
  - Chr 1p: disomy
  - Chr 3: disomy
  - Chr 6: disomy
  - Chr 8: disomy
- MSA: Our microsatellite assay (MSA) showed no loss of heterozygosity at any of the informative chromosome 3 loci tested, indicating a normal 2-copy result for chromosome 3.

Fig. 2. Excerpts from test result reports from Impact Genetics from a biopsy of uveal melanoma tissue with high-risk phenotype.

Important Caveats for Molecular Prognostication of Uveal Melanoma

1. The science and practice of prognostic testing in uveal melanoma seems to be less straightforward than previously believed. First, genetic heterogeneity within uveal melanoma tumors as detected with FISH and MLPA technique seems relatively common,[85–89] bringing into question the validity of a single biopsy result using these methods. A purported advantage of GEP testing is reliability of result despite tumor genetic heterogeneity as the test measures RNA in solution that may be expected to be more homogenous within a tumor than intracellular DNA/chromosomes. However, recent data have suggested that significant discordance between the GEP results from two consecutive biopsies of the same tumor may exist (11%–16%).[81] Limited data suggest that, if two biopsies of the same tumor have discordant GEP results, their prognosis seems similar to those with Class 2 profile. A proposed theory to explain this phenomenon is that low-metastatic potential uveal melanoma cells transform into high-metastatic potential cells over time, and a partial population of high-risk cells within a tumor may be all that is needed to cause distant tumor metastasis. In any case, this suggests that a single-site fine-needle aspiration biopsy with a low-risk result may be misleading in a minority of patients.[81]
2. Multiple studies have shown that certain morphologic characteristics of melanoma tumors (size and location) independently predict metastasis, beyond the data provided by either chromosomal analysis[15,48] or GEP.[16,17] We recommend that clinicians take into account the entire clinical picture, including tumor size, location, and tumor growth velocity when interpreting the results of genetic analysis of an individual uveal melanoma tumor.
3. It is important to consider actuarial data when determining prognosis from uveal melanoma as an older patient with significant comorbidities and a genetically poor-prognosis uveal melanoma may be more likely to die of a cause other than their melanoma.[90]
4. The Decision-Dx UM test does not provide diagnostic information and may provide a result even if the tumor biopsied is not a melanoma. For example, this test has provided a class designation in samples of choroidal metastases,[77,78] and these results are likely meaningless. The Impact Genetics Inc. test uses direct sequencing of GNAQ/GNA11 genes in an attempt to confirm that sampled tissue is indeed uveal melanoma but, as previously mentioned, data suggest that nonuveal melanoma tissue may also harbor this mutation.[40,75,76] Altogether this suggests that, in addition to a prognostic genetic tumor biopsy, concomitant biopsy for cytopathology may be appropriate to confirm the diagnosis of uveal melanoma in some patients, especially in less diagnostically certain cases. Unfortunately, FNAB for cytopathologic diagnosis is significantly less sensitive and specific than is FNAB for prognosis, often with insufficient tissue for analysis and/or producing of an indeterminate result.[14,56] Cytopathology testing is also not without risk. The amount of uveal melanoma tissue extracted during a single pass with fine-needle aspiration is not usually sufficient for both prognostic and cytopathologic tests. Therefore, two biopsies are required if both tests are desired. Each FNAB into uveal melanoma likely carries independent risks (see below), constituting an additional barrier to routine cytopathologic testing.

5. Extraocular extension of uveal melanoma is very rare but has been reported after biopsy of uveal melanoma (although some reported cases were after pars plana vitrectomy and open tumor biopsy instead of FNAB).[91–94] This can be avoided by various techniques that have been described including performing a peritomy over the biopsy site (so as not to sequester tumor tissue subconjunctivally), using a small gauge instrument (25 or 27 gauge), maintaining a dry/bloodless field during biopsy, using a *trans*-scleral cannula to create a protected needle tract,[95] and treating the sclerotomy (and any visible tumor tissue) with *trans* scleral cryotherapy. However, because of this possibly disastrous risk, performing a tumor biopsy after radiotherapy of uveal melanoma (instead of before) has some appeal (see below).
6. Limited data suggest that prognostic tumor biopsy performed with a 25-gauge vitreous cutter immediately after proton beam radiotherapy and using MLPA/MSA for analysis seems to be accurate at predicting metastasis.[96] Other data suggest that analysis of uveal melanoma tissue obtained through enucleation or endoresection at a long interval after proton beam radiotherapy or Ru^{106} brachytherapy produces similar results to biopsies of those same tumors performed before radiation.[97] Alternatively, karyotyping and FISH seem to be significantly less accurate in uveal melanoma after radiotherapy.[98] Two cases of a gene expression profile result being produced from a fine-needle aspiration biopsy of a choroidal melanoma long after treatment with iodine brachytherapy have also been reported (one with a Class 1 result and the other with a Class 2 result), but no data regarding the development of metastases on follow-up were reported, so no data support the accuracy of such a practice.[99] Indeed, according to their website, the Castle Biosciences, Inc. GEP test "cannot be run on radiated tissue."[22]
7. The accuracy of genetic analysis on formalin-fixed, paraffin-embedded tissue is a relevant question in case enucleation is the primary treatment for uveal melanoma and genetic analysis is preferred to be performed after tissue fixation. Data suggest that formalin-fixed, paraffin-embedded uveal melanoma tissue may be readily amenable to accurate prognostic testing with CGH/SNP and FISH,[100] but may reduce accuracy of MLPA when compared with snap-frozen tissue.[101] Although the initial studies validating gene expression profiling included many formalin-fixed, paraffin-embedded specimens, only limited data exist that the commercial GEP Decision-Dx test may be performed accurately on formalin-fixed, paraffin-embedded tissues.[102] The age and preservation state of the tissue may also limit the testing yield.

Conclusions

Predicting metastasis through evaluation of uveal melanoma genetics has come a long way since its first use 30 years ago, and biopsy/testing is now the standard-of-care for most patients. The technique of FNAB should be performed safely and, in combination with a commercially available genetic analysis test, seems to be reliable for uveal melanoma prognosis in most patients.

Although not widely available now, chemotherapy agents for metastatic uveal melanoma are being evaluated for their effectiveness. Once identified, such agents may be administered to patients before the development of radiologically detectable metastases. It is likely that the candidacy for such treatments will be based on the molecular signature of a patient's uveal melanoma, underscoring the importance of prognostic testing in this disease.

As advances in molecular genetics techniques are applied to uveal melanoma, our ability to predict metastasis may be more accurate and performed potentially on smaller samples. Looking forward, it seems that the genetic signature of circulating melanoma cells may correlate well with genetic data from tumor biopsy.[103] Although the technology to detect such cells is in its infancy, such noninvasive techniques may eventually supplant tumor biopsy for uveal melanoma prognostication.

Ramifications for Clinical Practice

1. Patients with uveal melanoma should be offered prognostic genetic testing of their tumor prior to treatment and when tumor biopsy is appropriate. The result may guide both the patient in life planning and the clinician in the timing of metastatic surveillance or adjuvant treatments.
2. Unless a practitioner has access to a laboratory for molecular analysis of biopsy specimens, only two commercially available platforms are currently available for prognostic analysis of uveal melanoma samples.
3. Both the Castle Biosciences Inc, and Impact Genetics Inc, tests may be performed on fine-needle aspiration samples. No data exist comparing these methods but, based on existing literature, both are likely quite accurate (the former has prospective data revealing the accuracy of that specific test, the latter has no prospective data but is based on genetic analysis techniques which have been validated over many years).
4. Prognostic biopsies are not equivalent to biopsies. Existing tests may produce a (meaningless)

diagnostic "result" even if the tumor biopsied is not a melanoma. If the diagnosis is truly in question, it is common practice to perform a diagnostic biopsy before tumor treatment through FNAB, direct *trans*-scleral open tumor biopsy or *trans*-vitreal biopsy with the vitreous cutter. When performing FNAB for genetic analysis, some centers also routinely perform a separate FNAB on all tumors for cytology as well.

5. Only emerging data exist regarding the accuracy of prognostic tumor biopsy of uveal melanoma through FNAB after radiotherapy.
6. Extraocular extension of uveal melanoma is rare but has been reported after biopsy of uveal melanoma.[91–93] Therefore care, experience, and expertise are essential when approaching biopsy of these malignant tumors.

Key words: uveal melanoma, choroidal melanoma, prognostication, biopsy.

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