Cytogenetic analysis of uveal melanomas: a long-term experience

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ABSTRACT:

**Background/aim:** Cytogenetic profile of posterior uveal melanoma (mainly monosomy 3) is actually considered the most specific prognostic factor for uveal melanoma patients. Nevertheless, there is still no consensus on which cytogenetic analysis should be used, and there is still no long-term data about the safety of sampling procedure. The aims of this study were to evaluate (i) long-term safety and efficacy of in-vivo 25-gauge transcleral Fine Needle Aspiration Biopsy (FNAB) and (ii) predictive value of Fluorescent In-situ Hybridization (FISH) vs Multiplex Ligation-dependent Probe Amplification (MLPA) analysis for cytogenetic testing of posterior uveal melanoma.

**Methods:** One hundred thirty-nine consecutive patients affected by posterior uveal melanoma with tumour thickness > 3mm underwent in-vivo 25-G transcleral FNAB (through the tumor base) just before applying the I-125 active plaque. A double pass sampling was performed. Sampled material underwent both FISH (chromosome 3 and 6) and MLPA analysis using standard procedures. Follow-up examination, including A/B-Scan eye and orbit ultrasonography, was performed after 1 month and every 6 months thereafter. Follow-up was longer than 24 months.

**Result:** Follow-up was 54±16 months (range, 24-84 months). FNAB yielded sufficient material for FISH analysis in 117 cases (84.2%). Fifty-six cases had monosomy 3 (47.9%). No clinically relevant monosomy 3 heterogeneity was detected (double pass sampling). Chromosome 6 co-detection using FISH was performed in forty-four patients. Monosomy 3 and +6p resulted mutually exclusive in 40 cases (90.9%). Univariate Cox analysis showed metastatic disease to be strongly associated with monosomy 3 (p=0.005). No misclassification occurred in
low risk patients having both disomy 3 and +6p. MLPA was performed in twenty-four patients revealing monosomy 3 in thirteen cases (54%) (vs twelve cases classified by FISH) and a 3p14-q29 deletion in one case (4%) (classified as monosomy 3 by FISH). Considering this sub-group of twenty-four patients having both FISH and MLPA, nine patients (41%) developed metastatic disease during follow-up, including the case showing monosomy 3 only by MLPA. Patient with partial chromosome 3 deletion by MLPA is still alive without metastases. Due to FNAB procedure, three patients developed transient, localized and self-limited subretinal haemorrhages after FNAB. Neither other short- and long-term complications nor extrascleral extensions were documented during follow up.

**Conclusion:** The use of 25-G transcleral FNAB appears a long-term safe and effective procedure for in-vivo cytogenetic testing of posterior uveal melanoma. Combined analysis of both arms of uveal melanoma bifurcated pathway (-3 and +6p) increase predictive value of FISH technique. MLPA allows obtaining more information than standard FISH in uveal melanoma prognostication. The biological and prognostic value of partial chromosome 3 deletion, as well as others subtle chromosomes alterations or complex MLPA results, remains unclear.
RIASSUNTO:

**Introduzione/scopo:** il profilo citogenetico del melanoma uveale (in particolare la monosomia 3) è attualmente considerato il fattore prognostico più specifico per il rischio metastatico nei pazienti affetti da melanoma uveale. Ciononostante, non c’è ancora accordo su quale analisi citogenetica debba essere preferita. Inoltre, mancano dati a lungo termine sulla sicurezza della procedura di prelievo. Gli obiettivi di questo studio sono (i) valutare la sicurezza a lungo termine e l’efficacia del prelievo transclerale in-vivo mediante citoaspirazione con ago sottile (FNAB) da 25-gauge e (ii) valutare il valore predittivo del metodo Fluorescent In-situ Hybridization (FISH) vs il metodo Multiplex Ligation-dependent Probe Amplification (MLPA) per l’analisi citogenetica del melanoma coroideale

**Materiali e metodi:** Centotrentanove pazienti, consecutivamente reclutati poiché affetti da melanoma uveale con spessore tumorale >3 mm, sono stati sottoposti a prelievo in-vivo mediante FNAB transclerale con ago da 25-gauge (attraverso la base del tumore), immediatamente prima di suturare la placca radiante di I-125. Il prelievo è stato ripetuto due volte. Il materiale prelevato è stato sottoposto a FISH (cromosomi 3 e 6) e MLPA usando le procedure standard. Il protocollo di follow-up, incluso l’ecografia A-B scan del bulbo oculare e dell’orbita, prevedeva una visita al primo mese dopo l’intervento e, in seguito, ogni 6 mesi. Il follow-up minimo è stato superiore a 24 mesi.

**Risultati:** Il follow-up medio è stato di 54±16 mesi (range, 24-84 mesi), La procedure FNAB ha fornito sufficiente materiale per l’analisi FISH in 117 casi (84.2%). Cinquantasei casi sono risultati monosomici per il cromosoma 3 (47.9%). Non è stata rilevata eterogeneicità clinicamente rilevante della monosomia 3.
all'interno dello stesso tumore (doppio prelievo). La co-analisi del cromosoma 6 è stata effettuata in quarantaquattro pazienti. La monosomia del cromosoma 3 e il +6p sono risultate mutualmente escluenti in 40 casi (90.9%). Lo sviluppo di malattia metastatica è stato correlato in modo significativo con la presenza della monosomia 3 (p=0.005) (Univariate Cox analysis). Nessun paziente a basso rischio, caratterizzato sia da disomia 3 che da +6p ha sviluppato malattia metastatica. L'analisi MLPA è stata eseguita in ventiquattro pazienti, rilevando la presenza di monosomia 3 in tredici casi (54%) (vs dodici casi classificati monosomici dalla FISH) e la presenza di una delezione 3p14-q29 in un caso (4%) (classificato come monosomico dalla FISH). Considerando questo sottogruppo avente sia il dato FISH che MLPA, nove pazienti (41%) hanno sviluppato malattia metastatica, compreso il caso classificato come monosomico solo dall'analisi MLPA. Il paziente con delezione parziale del cromosoma 3 è ancora in vita senza segni di malattia metastatica in atto. Tre pazienti hanno presentato una limitata emorragia vitreale spontaneamente regredita in pochi giorni in seguito al prelievo FNAB. Nessun'altra complicanza a breve o lungo termine, compreso lo sviluppo di estensione extrasclerale della neoplasia in sede di prelievo, è stata rilevata durante il follow-up.

**Conclusioni:** L'uso del prelievo FNAB transclerale mediante ago da 25-gauge è una procedura sicura ed efficace per l'analisi citogenetica del melanoma uveale. L'analisi combinata di entrambe le vie patogenetiche (-3 e +6p) innalza il valore predittivo della tecnica FISH. La tecnica MLPA consente di ottenere maggiori informazioni rispetto alla tecnica FISH. Purtroppo però, il valore biologico e prognostico di delezioni parziali del cromosoma 3 o di altre alterazioni cromosomiche parziali rimane sconosciuto.
INTRODUCTION

Posterior uveal melanoma (UM) is the most common primary intraocular malignant tumor in adults, with an annual incidence of five to seven cases per million population (FIG1).\(^1\) Although the goal of treating the tumor and preserving the eye is achieved in most cases (>90% using eye preserving radiation treatments), overall mortality remains high (about 50%).\(^2\) Nevertheless, less than 2% of affected patients have clinically detectable metastasis at presentation.\(^2\) The preferred spread of UM is haematogenous, and the liver is often the first and prevalent site of metastatic disease (FIG2).\(^3\) However, UM can metastasize to any organ and other common sites include the lungs, bones, soft tissues, gastro-enteric tract, ovaries, kidneys and central nervous system (CNS).\(^3\) The reported median life expectancy of patients affected by metastatic UM ranges from 3.6 to 15 months, with worse survival in larger series and better survival in the smaller ones.\(^4\) Site of metastases, number of metastatic sites, percentage of liver substitution, diameter of liver metastases, presence of symptoms, alteration of liver function tests, especially alkaline phosphatase (ALP) and lactic dehydrogenase (LDH), older age, male sex, and a shorter metastasis-free interval have been associated with a poorer prognosis.\(^3\) Before the introduction of eye preserving treatment based on irradiation of the tumor, eye enucleation was the standard treatment for UM patients, and histopathologic characteristics of the tumor were considered the most reliable prognostic factor.\(^5, 6\) Because most UM are currently treated with conservative treatment, no material is available for cytologic or histologic evaluation.\(^6\) Therefore, most treated patients receive prognostic information based only on clinical characteristics of the tumor (largest basal diameter, thickness, location).\(^2, 6\) Unfortunately, these parameters are unable to accurately characterize patients’
It has been recently demonstrated that a cytogenetic characteristic of UM, namely monosomy 3 (-3), is highly predictive of metastatic disease.\(^2\),\(^5\)-\(^6\) This parameter seems a better predictor of metastasis than any other clinical and/or histopathologic parameter previously reported.\(^7\) Some retrospective analyses of cytogenetic data have hypothesized that two cytogenetic pathways of clonal evolution exist in UM: one starts with the loss of an entire chromosome 3 and continues with gains of 8q; the second pathway starts with gain of 6p (FIG 3).\(^8\)-\(^11\)

Long-term studies have shown that about 70% of patients with monosomy 3 in the primary tumor died from metastases within 4 years after the initial diagnosis, whereas tumors with normal chromosome 3 status (disomy 3) rarely gave rise to metastatic disease (FIG 4).\(^2\),\(^5\)-\(^6\),\(^8\)-\(^11\) Therefore, monosomy 3 is considered a highly specific marker for poor prognosis in UM.\(^2\),\(^5\)-\(^6\),\(^8\)-\(^11\) Monosomy 3 has been initially detected only in enucleated eyes or histologic specimens from resected tumors.\(^2\),\(^5\)-\(^6\)

Therefore, patients treated with irradiation (90% of UM population) were excluded from this prognostic information. Considering that accurate identification of patients at high risk of developing metastatic disease may be relevant for clinical management of any UM patients, few years ago, our team have first described the in-vivo sampling technique for cytogenetic testing of UM conservatively treated.\(^2\),\(^5\)

After our first report, many centres started performing tumor biopsy for UM prognostication, using different sampling techniques and different cytogenetic/molecular biology analysis.\(^11\) Moreover, there is still no consensus on which sampling technique and cytogenetic analysis should be used, and there is still no agreement on the overall interpretation of the interrelationships between chromosomes changes (mainly chromosome 6 and 8) and their prognostic values.\(^11\) Moreover, there is still no long-term data about the safety of different sampling procedure.
AIM

The aims of this study were to evaluate (i) long-term safety and efficacy of in-vivo 25-gauge transcleral Fine Needle Aspiration Biopsy (FNAB) and (ii) predictive value of Fluorescent In-situ Hybridization (FISH) vs Multiplex Ligation-dependent Probe Amplification (MLPA) analysis for cytogenetic testing of posterior UM.

MATERIALS AND METHODS

This study complied with the tenets of the Declaration of Helsinki, and was approved by the IRB of our institution. Patients were recruited from those referred to the Ophthalmic Oncology Unit of the Department of Ophthalmology, University of Padova, Padova, Italy, between September 2001 and November 2009. Each patient underwent full preoperative ophthalmic and systemic examination. The ophthalmoscopic aspects of the tumors were documented using fundus photography (FIG1). UM dimensions were also documented using A- and B-scan ultrasonography. Liver enzymes and liver ultrasonography were used to evaluate the presence of metastatic disease at baseline. Eligible patients were affected by primary UM with tumour thickness > 3mm, and were free of metastasis and other cancers at enrolment, free of melanoma extrascleral extension, 21 years or older, and free from other life-threatening coexisting systemic diseases. Informed consent was obtained after explanation of the nature of the disease and the possible implications of this study. All patients were scheduled for standard plaque brachytherapy with I125, under general anaesthesia. One hundred thirty-nine consecutive patients undergoing plaque radiotherapy, with follow-up longer than 24
months, were included in this study. Patients underwent an in-vivo intraocular FNAB during the procedure for radioactive plaque application, as previously described (Fig 5-8). Briefly, the cytologic sampling was made through the sclera, just before suturing the active I125 plaque over the tumor base. A standard FNAB procedure was performed using a 25-gauge blunt needle, 27 mm in length (Becton Dickinson, New York, NY, USA). A 200–300 µ pre-planned scleral incision was made, and the needle inserted into the tumor through the remaining sclera. A double-pass sampling was performed. The scleral incision was sutured with 7.0 Polyglactin suture, and the radioactive plaque immediately placed over the tumor base. Tumor specimens obtained by FNAB were collected in culture medium RPMI 1640 (Euroclone Life Science, Milano, Italy). Each patient was treated by Iodine-125 plaque brachytherapy following the American Brachytherapy Society recommendations for brachytherapy of UM (dose of 85-100 Gy at tumor apex, dose rate of 0.60–1.05 Gy/h). Each patient underwent a 1-month follow-up examination, and every 6 months thereafter. Follow-up examination included full ophthalmologic examination, fundus photography and A- and B-scan ultrasonography. Orbit nuclear magnetic resonance was used in selected cases basing on clinical and ultrasonography data. Liver enzymes analysis and liver ultrasonography were also performed every 6 months to check for metastatic diffusion. When the site of needle aspiration was detectable through slit lamp examination, this area underwent regular photographic follow-up.

FISH for chromosome 3 was performed with a centromeric probe labeled with SpectrumOrange fluorocrome (Vysis-Abbott, Downers Grove, IL, USA), as previously described by our group (Fig 9-10). Briefly, after sedimentation, the material was enzymatically digested with collagenase II (Worthington, NJ, USA) 1400 U/mL at 37 °C for 2 hours. Then the suspension was washed in RPMI 1640
and used to prepared cytospins. Slides were fixed with a cytologic fixative (Bio- Fix; Bio-Optica, Milano, Italy), and stored at –20 °C. FISH analysis was performed with a centromeric probe for chromosome 3 labeled with SpectrumOrange and centromeric probe for chromosome 10 labeled with SpectrumGreen (Abbott-Vysis, Downers Grove, IL, USA) following the manufacturer’s procedure. Slide and probe were codenatured in Hybrite’ (Vysis) at 75 °C for 5’ and hybridized in a humid chamber overnight at 42 °C. Post-hybridization washes were made at 73 °C in 0.4 x SSC/0.3% NP-40 for 2’ and at room temperature in 2 x SSC/0, 1% NP-40 for 1’. Slides were air dried and mounted with a Vectashield, mounting medium with DAPI (Vector Laboratories, Burlingame). Microscope analysis was carried out with a fluorescent microscope (Zeiss Axioplan fluorescent microscope, Germany) equipped with a cooled charge-coupled device (CCD) camera (Hamamatsu, Hamamatsu-city, Japan) and appropriate single band and triple band filters. Images were analyzed using CRO- MOFISH software (Amplimedical, Assago-MI, Italy). At least 100 cells were evaluated for each case; loss of chromosome 3 was reported when more than 15% of cells showed a single signal for chromosome 3.

FISH analysis for chromosome 6 was carried out with BAC clone RP11-513I15 located at 6p21.31 and RP11-697G4 for 6q located at 6q21 obtained from M. Rocchi (Resources for Molecular Cytogenetics, Bari, http://www.biologia.uniba.it/rmc/), as previously described by our group (Fig 9-10). Briefly, BAC probes were biotin- and digoxigenin-labeled by nick translation using the biotin-nick and DIG-nick translation mix (Roche, Mannheim, Germany). For each experiment, 100ng of labeled probe were used for hybridization following standard procedures. Signal detection was made using Avidin-Cy3 (Amersham Biosciences, Little Chalfont, UK) diluted 1:100 in phosphate-buffered saline (PBS; Gibco-BRL, Paisley, UK) and anti-digoxigenin–fluorescein (Roche) diluted 1:200 in
PBS. Microscopic analyses were carried out using a fluorescent Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany) with appropriate single band and triple band filters. At least 100 cells per sample were evaluated and a cut-off of 10% for chromosome 6 probes was considered. Results of hybridization with 6p (red) and 6q (green) probes were classified as follows: a signal pattern of 3R1G and 4R1G was considered as +6p.

MLPA analysis was carried out using a kit specifically designed for UM (SALSA P027 (B1; MRC-Holland), as previously described (Fig 11). This comprises 12 control probes and test probes directed at 7 loci on chromosome 1, 13 loci on chromosome 3, 6 loci on chromosome 6, and 5 loci on chromosome 8. DNA extraction, DNA quality assessment and quantification, and MLPA were conducted as previously reported.  

RESULTS

One hundred thirty-nine consecutive patients affected by posterior UM (mean age, 63.3 ± 10.4 years; range, 36 to 82 years) were included in this study. Seventy-five patients were male (56.2%) and sixty-four female (43.8%). The right eye was affected in 71 patients (50.7%) and the left eye in 68 cases (49.3%). Ninety-seven melanomas were purely choroidal in location (70.3%) whereas 42 eyes were affected by ciliary body tumors (29.7%). Mean tumor largest basal diameter was 12.5 ± 2.7 mm (range, 7–15 mm) and mean tumor thickness was 6 ± 2.9 mm (range, 3–11 mm). Mean follow-up was 54±16 months (range, 24-84 months). Transcleral FNAB yielded enough material for (at least) a single FISH analysis (monosomy 3) in 117 cases (84.2%). In the remaining 21 cases, the aspirate was graded as insufficient (15.8%). FNAB yielded sufficient specimens in 39 ciliary body
tumors (92.8%) and in 79 choroidal neoplasms (81.4%) (p=0.037). No correlation was found between tumor dimension (thickness and largest basal diameter) or tumor location (ciliary body vs choroidal location) and adequacy of FNAB (p > 0.05). Monosomy 3 was detected in 56 cases (47.9%) and disomy 3 in the remaining 61 cases (52.1%). No correlation was found between chromosome 3 status and tumor dimensions (p> 0.05), and between monosomy 3 and tumor location (ciliary body vs choroidal location) (p > 0.05). Among monosomy 3 tumors, the mean percentage of monosomic cells in each sample was 89% ± 9.4% (range, 39–100%). Considering a cut-off of 15%, no clinically relevant monosomy 3 heterogeneity was detected separately analysing sampled material obtained by the double pass sampling procedure. Considering patients with monosomy 3 (N=117), thirty-one patients (26.5%) died during follow up. Twenty-nine of them (93.5), died due to liver metastatic disease, which developed 24 ± 7 months (range, 16–40 months) after treatment, without any evidence of extrascleral recurrence in the sampled site. Two patients (6.5%) died of unrelated causes. Considering the 29 patients that developed metastatic disease, 22 were affected by monosomy 3 UM, whereas 7 by a disomy 3 tumor. Univariate Cox analysis showed metastatic disease to be strongly associated with monosomy 3 (p=0.005).

Chromosome 6 co-detection using FISH was performed in forty-four patients. Chromosome 6 normal pattern was found in 24 patients (55.6%), whereas 20 patients were affected by a +6p tumor (45.4%). Monosomy 3 and +6p resulted mutually exclusive in 40 cases (90.9%), whereas the coexistence of -3 and +6p was found in 4 patients (9.2%). Considering this sub-group having both chromosome 3 and 6 data, 12 patients (28.5%) developed metastatic disease. Of these, 3 patients have both -3 and +6p (25%), and 9 patients were affected by monosomy 3 tumors with normal chromosome 6 (75%). No misclassification occurred in low risk patients
having both disomy 3 and +6p (no metastatic disease). MLPA was performed in twenty-four patients having also FISH result for chromosome 3. MLPA revealed the presence of monosomy 3 in thirteen cases (54%) (vs twelve cases classified as -3 by FISH) and a 3p14-q29 deletion in one case (4%) (classified as monosomy 3 by FISH) (Fig 11). Considering this sub-group of twenty-four patients having both FISH and MLPA results, nine patients (41%) developed metastatic disease during follow-up, including the case showing monosomy 3 only by MLPA. Patient with partial chromosome 3 deletion by MLPA is still alive without metastases.

DISCUSSION

Ophthalmic oncologists are among the few, if not the only, cancer-treating physician who do not routinely use cyto- or histologic confirmation before treating a clinically diagnosed malignancy.\textsuperscript{11} Therefore, intraocular FNAB is not usually used in a routine clinical setting to sample intraocular lesions.\textsuperscript{11,15} There are at least two reasons: a wide accepted high accuracy in non-invasive diagnosis of posterior UM by expert clinicians, and the claimed risk of tumor diffusion secondary to diagnostic invasive approaches.\textsuperscript{11} About the former, it has been convincingly proven by the Collaborative Ocular Melanoma Study (COMS) for lesions whose dimension are thickness $>3$ mm and largest basal diameter more than 10 mm.\textsuperscript{16} But about the latter, there is no evidence of an increased risk of UM local diffusion following correctly performed FNAB.\textsuperscript{11} Nevertheless, complications are typically under-reported in literature and, to the best of our knowledge, no specific long-term studies are available on this topic. Moreover, UM shows some peculiarities compared to the majority of solid cancers: (i) disease related mortality is unchanged
during the last 100 years, despite the introduction of new conservative therapeutic approaches [7]; (ii) UM mortality seems completely unrelated to type and result of local treatment (not conclusive evidence of a life-extending benefit of local treatment for medium-size UM) (iii) cytogenetic profile of posterior UM is extremely simple (unlike of most solid tumors) and is currently considered the most important prognostic factor for metastatic disease. These considerations give raise relevant questions about the biology and the natural history of UM, moving the interest of the clinicians from the treatment of the primary tumor to its biological behaviour.

Cytogenetic analysis of posterior UM has contributed to the delineation of the pattern of genetic alterations in this tumor. Recent chromosomal abnormalities affecting chromosome 1, 3, 6 and 8 have been described and correlated with prognosis. Loss of chromosome 3, loss of short arm of chromosome 1 and gain of 8q have been associated with decreased survival, whereas +6p with a low metastatic risk. Because histologic material is not traditionally available when conservative treatment is performed, conservatively treated patients were excluded from any information about life prognosis. We considered that moving cytogenetic prognostication from the ex-vivo to the in-vivo arena may represent a relevant improvement in the clinical identification of this risk factor in patients affected by posterior UM. Moreover, clinicians are unable to accurately select patients at high risk for metastasis to be included in on-going studies of adjuvant chemotherapy basing only on clinical UM features. These considerations led us to introduce, few years ago, a simple and reliable technique to detect cytogenetic alterations in-vivo. With this long-term study we have demonstrate that early complications following intraocular FNAB are rare and time-limited. Moreover, we have never found any long-term complication, as well as local recurrences or extra-ocular extension due to sampling procedure. To the best of our
knowledge, only one suspected clinical case of extrascleral recurrence has been reported after diagnostic intraocular transscleral FNAB in UM patients. Furthermore, compared to our approach that includes full radiation treatment of the entry site, no treatment was applied over the sampled area and the tumor base. Thus, concerns about tumor seeding due to this diagnostic procedure should be dispelled by our data. The proportion of adequate biopsies for analysis was higher for ciliary body melanomas than for choroidal melanomas, reaching statistical significance. This fact may be explained by the higher technical difficulties of sampling more posteriorly located tumors. A transvitreal approach has recently been investigated for these lesions by Shields et al. According to this study, based on 140 eyes with limited follow-up, tumors located posterior to the equator (67 of 140 eyes: 48%) and sampled via pars plana yielded sufficient material in 65 of 67 cases (97%). Unfortunately, this study reports a high number of local complications (vitreous haemorrhages in 64 eyes), probably related to the direct trans-retinal approach to the tumor apex. In the same series, a lower rate of sufficient material (75% vs 97%) was obtained when the tumors were sampled with 30- vs 27-gauge needle. Balancing needle diameter vs tumor approach needs further investigation. A 25-gauge vitrector has recently been claimed to give more material than transvitreal or transscleral FNAB. Unfortunately, this technique is more aggressive and surgically demanding, and its safety is unknown. Moreover, using the FNAB technique, a double-pass is always recommended to reduce the incidence of insufficient sampling. Sampled tumors were not classified by cytology after FNAB, because FNAB material was fully used for cytogenetic analysis. FNAB adequacy, as reported by Sisley et al., seems independent of melanoma dimensions, as confirmed by our data. FISH testing showed monosomy 3 in 47.9% of our cases. Previous ex vivo studies reported monosomy 3
in 40–65% of sampled cases.\textsuperscript{9–20} The high percentage of monosomy 3 cells in each positive sample (89% ± 9.4%) shows that, when present, monosomy 3 is fully exposed and easily detected by FNAB sampling. The presence of heterogeneous distribution of monosomy 3 has been recently evaluated in some case series, with conflicting results.\textsuperscript{21} However, Meir et al., using a larger pattern of laboratory investigations, were not able to detect tumor heterogeneity.\textsuperscript{21} In our study no correlation was found between monosomy 3 and tumor dimension. Considering literature data, the existence of a correlation between these two parameters remains unclear.\textsuperscript{2, 6, 18, 22} Our sampling technique for in-vivo cytogenetic prognostication of posterior UM differs from standard FNAB.\textsuperscript{2, 6} We use partial scleral incision, followed by suture immediately after sampling, to avoid excessive pressure when penetrating the sclera (particularly relevant in small tumors). This manoeuvre, followed by direct application of radioactive plaque over the tumor base, improves the safety of tumor sampling.\textsuperscript{11} Long-term safety of this sampling procedure is proven by the absence of any local extrascleral recurrence during a log-term follow-up. Moreover, patients’ mortality rate from liver metastasis 36 months after treatment agrees with previously published data.\textsuperscript{3} Our data demonstrate that monosomy 3 alone sometimes fail to personalize patients prognosis, mainly considering the false-negative results (patients without monosomy 3 that will develop metastatic disease). To reduce false negative results we have introduced the chromosome 6 co-detection, aimed exploring the second cytogenetic pathway of UM.\textsuperscript{13} This double check can be performed using a second FISH analysis or MLPA, exploring in a single reaction different selected chromosomes related to UM pathway and prognosis.\textsuperscript{13} Using chromosome 6 co-detection by FISH we have reduced the false-negative results because, considering the sub-group having both chromosome 3 and 6 data, no misclassification occurred
in low risk patients having both disomy 3 and +6p (no metastatic disease).
Therefore, we retain that a multichromosomal analysis should be actually preferred.

Considering MLPA vs FISH analysis, MLPA revealed a single case of monosomy 3 that FISH classified as non-monosomic. Considering that this patient developed metastatic disease during follow-up, we conclude that MLPA is superior to FISH in the cytogenetic prognostication of UM, even if the difference in specificity is minimal. Another advantage of MLPA is that using one single reaction this test can explore more than a single chromosome status, allowing checking both UM pathways in a single reaction. About the sampling amount of material for both FISH and MLPA, in our experience, these two techniques are in need of the same amount of cells. Moreover, the cost of these two analyses is similar. The second claimed advantage of MLPA is its ability to detect subtle structural chromosomal alterations compared to FISH. In our study only a single case of partial chromosome 3 deletion was found by MLPA (3p14-q29), which was classified as non-monosomic by FISH. We actually do not know the prognostic value of chromosome 3 partial deletions, considering that only monosomy 3 is claimed to be prognosis-related. In this case of 3p14-q29 deletion, the patients do not developed metastatic disease during follow-up. Thus, relevant question are rising on the biological means of partial chromosome 3 deletions, as well as their prognostic value. Recently, Harbour et al have suggested the presence of a prognosis-related minimal region of deletion in UM, located in 3p21.1 and encoding BRCA1-associated protein 1 (BAP1). The absence of BAP1 involvement in our patient with partial chromosome 3 deletion may explain the lack of metastatic disease during follow-up in this case. Unfortunately, no other group has confirmed this finding on BAP1. Therefore, we do not precisely know if a minimal region of deletion with prognostic value really exists in UM (and where is located on chromosome 3).
Thus, we retain that partial chromosome 3 deletions should actually not be considered clearly as a prognosis related alteration. Therefore, there are actually no many advantages, in a routine clinical setting, using MLPA compared to FISH to check for subtle chromosomes alteration: even if we get a lot more information by MLPA, we are not able to interpreter the biological and prognostic means of these findings.  

Other laboratory techniques have been used to investigate monosomy 3 in UM: single nucleotide polymorphisms, comparative genomic hybridization array and microsatellite analysis are claimed to be able to detect more subtle structural alterations.  

However, the same considerations of MLPA are applicable to these techniques. Moreover, FISH technique continues to be considered an appropriate and reliable method for UM prognostication, and it is easily available in many clinical laboratories. 

In conclusion, the use of 25-G transcleral FNAB appears a long-term safe and effective procedure for in-vivo cytogenetic testing of posterior UM. Combined analysis of both arms of UM bifurcated pathway (-3 and +6p) increase predictive value of FISH technique. MLPA allows obtaining more information than standard FISH in UM prognostication. The biological and prognostic value of partial chromosome 3 deletion, as well as others subtle chromosomes alterations or complex MLPA results, remains unclear.
FIGURE LEGENDS

Fig.1: Medium-sized uveal melanoma in a left eye.

Fig 2: Liver metastases from uveal melanoma.

Fig 3: Bifurcated cytogenetic pathways of UM clonal evolution: one starts with the loss of an entire chromosome 3 and continues with gains of 8q (high risk of metastatic disease); the second pathway starts with gain of 6p (low-risk).\textsuperscript{29}

Fig 4: Metastases-free survival probability related to monosomy 3 presence/absence.\textsuperscript{27}

Fig 5: Tumor trans-illumination aimed at correctly localize tumor base during sampling procedure.

Fig 6: Tumor localization after trans-illumination.

Fig.7: Tumor sampling using 25-gauge needle.

Fig.8: Sampled material is collected on RPMI. Note the large amount of tumor material in this case.

Fig 9: Fluorescence in situ hybridization analysis of tumor material sampled by fine needle aspiration biopsy. A case with disomy 3: each cell has two chromosomes 3 (in red) and two chromosomes 10 (labelled in green as controls).

Fig 10: Fluorescence in situ hybridization analysis of tumor material sampled by fine needle aspiration biopsy. A case with monosomy 3: each cell has two chromosomes 10 (labelled in green), but only one chromosome 3 (labelled in red).

Fig 11: Uveal melanoma with a 3p14-q29 deletion: MLPA and FISH comparison. Note that FHIS classified this patient as monosomy 3 (centromeric probe in included in the region of deletion).
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