Comparison of Formalin-Fixed and Snap-Frozen Samples Analyzed by Multiplex Ligation-Dependent Probe Amplification for Prognostic Testing in Uveal Melanoma

Sarah L. Lake,[1,2] *Helen Kalirai,*[1,2] *Justyna Dopierała,*[1,2] *Bertil E. Damato,*[3] *and Sarah E. Coupland*[1]

Purpose. Survival of patients with uveal melanoma (UM) correlates strongly with chromosomal abnormalities, such as monosomy 3 and polysomy 8q. Studies have shown multiplex ligation-dependent probe amplification (MLPA) to detect reliably chromosomal aberrations in UM using frozen samples. To date, the concordance between MLPA data generated from frozen and fixed samples has not been investigated. Our study aimed to compare MLPA data obtained from fixed and frozen tissues to determine whether fixed samples can be used in UM genetic prognostic testing.

Methods. Adjacent samples were taken from 20 UM cases, with one sample being snap-frozen, and the second being formalin-fixed and paraffin-embedded (FFPE). MLPA analysis was performed using the P027.B1 assay. The chromosome arm copy numbers obtained for each pair of UM samples then were compared.

Results. Of the 20 UM cases 18 gave MLPA results from FFPE material that passed quality control thresholds. When chromosome 3 copy number was classified successfully (loss, disomy or gain) using FFPE material, the corresponding frozen sample showed concordance in 100% of cases. However, even when FFPE samples passed quality thresholds, chromosome arm copy numbers for 3p and 3q were "unclassifiable" in 28% and 11% of cases, respectively. This compared to "unclassifiable" cases in 0% and 6% of the frozen UM samples.

Conclusions. Whenever possible, fresh or snap-frozen tissue should be used for UM genetic prognostic testing by MLPA. When only fixed tissue is available, MLPA can be used to determine reliably chromosome 3 copy number. However, some tumors will be unclassifiable where DNA quality is poor. (*Invest Ophthalmol Vis Sci.* 2012;53:2647–2652) DOI:10.1167/iovs.12-9584

From the [1]Department of Molecular & Clinical Cancer Medicine, University of Liverpool, Liverpool, United Kingdom, and [3]Liverpool Ocular Oncology Service, Royal Liverpool University Hospital, Liverpool, United Kingdom.

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[2]These authors contributed equally to this manuscript.

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Corresponding author: Sarah E. Coupland, Professor and Honorary Consultant in Pathology, Department of Molecular & Clinical Cancer Medicine, University of Liverpool, 6th Floor Duncan Building, Daulby Street, Liverpool, L69 3GA, UK; Telephone +44-151-706-5885; Fax:+44-151-706-5859; s.e.coupland@liverpool.ac.uk.

Almost 50% of all patients with uveal melanoma (UM) die of metastatic disease, which is detectable only rarely when the primary intraocular tumor is treated. There is scope for personalized prognostication, so that patients with a good survival probability can be reassured while targeting screening at high-risk patients. Metastatic disease occurs almost exclusively in patients whose tumor shows chromosome 3 loss and this abnormality is associated with a high mortality.

Several genetic methods have been used to estimate the survival probability of patients with UM.[1–4] For several years, we have used multiplex ligation-dependent probe amplification (MLPA) for prognostication and for deciding subsequent patient management.[1,2,5–7] MLPA tests 31 loci across chromosomes 1p, 3, 6, and 8 to detect chromosome arm copy number. Although less strongly associated with patient survival than monosomy 3 and polysomy 8q, gains of chromosome 6p are associated with good patient prognosis in UM and loss of chromosome 1p is associated with a poorer prognosis.[1,8-11] Studies have demonstrated the efficacy of MLPA for prognostication.[1,12] Whenever possible, we have used snap-frozen or fresh tissues; however, in some cases only formalin-fixed, paraffin-embedded (FFPE) tissue is available; for example, when analyzing archived samples obtained from patients treated months or years previously.

Tissue fixation for long-term storage has been shown to cause nucleic acid-protein cross-linking and nucleic acid degradation.[13–18] The detrimental effects of tissue fixation on molecular analyses are well documented.[19–22] However, by paying due attention to the limitations of such studies, it is possible to obtain useful results using fixed tissues.[21,23–25] Several studies have shown MLPA and methylation-specific MLPA to yield reproducible and reliable results using FFPE samples of a wide range of tumors, which include cutaneous melanocytic lesions,[26,27] gliomas,[28] breast carcinoma,[29] gastric cancer,[30] and UM.[31] Indeed, Moerland et al.,[29] and Buffart et al.,[30] demonstrated analogous results from FFPE tissues when MLPA was compared to fluorescence in situ hybridization or array comparative genomic hybridization.

To our knowledge, no direct comparison of MLPA data generated from fixed and unfixed tissues has yet been performed. The aims of our study, therefore, were to determine: (a) if chromosome arm copy number assessed in FFPE specimens of UM is comparable to that using unfixed samples, and (b) whether FFPE tissue samples are a reliable source of DNA for routine prognostic genetic testing in UM.

Materials and Methods

Samples

We selected for this study 20 patients undergoing enucleation ($n = 15$) or local resection ($n = 5$) of UM between late 2009 and early 2010 at

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the Royal Liverpool University Hospital (RLUH). Patients had received no other treatment preoperatively. Immediately after such surgery, two approximately equal-sized and adjacent samples were taken from the same area of each UM: one tumor sample was snap-frozen immediately in liquid nitrogen and stored at −86°C, and the second sample was fixed in 10% neutral-buffered formalin for 24-hours before being processed for embedding in paraffin according to standard protocols within the RLUH Histopathology Department. The same procedure was applied to six samples of normal choroid obtained from six enucleated eyes, which served as normal controls for MLPA.

After harvesting tissues for research purposes, all tumors were fixed in formalin, and processed through to paraffin embedding, for routine diagnostic reporting. The diagnosis of melanoma was confirmed with hematoxylin and eosin (H & E) stained sections and, if necessary, using Melan A immunostains. Spindle and epithelioid cell types were identified using the modified Callendar system.[32] Extravascular matrix patterns were assessed as described previously using the PAS reagent, without counterstaining.[2,31] The mitotic count was obtained by documenting the number of mitoses in 40 high power fields (HPF) using the 40× objective.[2,31]

Informed consent was obtained from each patient and research was performed according to the Tenets of the Declaration of Helsinki. Ethical approval was obtained for this study from the Local Research Ethics Committee (LREC number 01/103).

Multiplex Ligation-Dependent Probe Simplification

DNA extraction, DNA quality assessment and quantification, and MLPA were performed as reported previously, and are described briefly below.[2,31]

DNA Extraction from Frozen Tissue

Tissues were lysed in 400 µL TSE buffer (10 mM Tris/HCl [pH 7.5], 0.4 M NaCl, 2 mM EDTA [pH 8.0]), 0.9% SDS, and 61.5 mg proteinase K (1.64 mg/µl; Qiagen GmbH, Hilden, Germany) with incubation overnight at 56°C (Eppendorf Thermomixer Comfort; Sigma-Aldrich, Gillinham, UK). A total of 61.5 mg of proteinase K was added after 16 hours and samples were incubated for a further 4 hours. Genomic DNA was isolated by salt (5 M NaCl) and ethanol precipitation, and DNA dissolved in 20 to 100 µL of TE buffer (10 mM Tris [pH 8.5], 1 mM EDTA [pH 8.0]), depending on the size of the DNA pellet.

DNA Extraction from FFPE Tissue

Tissues were incubated for 16 hours at 56°C (Thermomixer Comfort, Eppendorf) in 125 µL of P-buffer (50 mM Tris-HCl [pH 8.2], 1 mM EDTA, 100 mM NaCl, 0.5% Tween-20, 0.5% NP40, 20 mM dithiothreitol) containing 8.2 mg of proteinase K (Qiagen). Samples were incubated further at 37°C, following the addition of 8.2 mg of proteinase K for 24 hours. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) and the DNA eluted in 40–50 µL of AE buffer.

DNA Quantification and Quality Assessment

DNA concentration was measured by the NanoDrop Spectrophotometer (Thermo-Fisher Scientific, Cambridge, UK) and its quality was assessed by multiplex PCR (adapted from van Dongen et al.[33]), as described previously by Dopierala et al.[31]

Multiplex Ligation-Dependent Probe Amplification

Using 200 ng of DNA per reaction, MLPA was performed in triplicate with the P027.B1 UM assay (MRC-Holland, Amsterdam, The Netherlands). Six non-tumor, normal choroid, control samples and a negative (no template) control were analyzed alongside the UM test samples in each reaction. MLPA was performed in a G-Storm GS1 Thermal Cycler (Genetic Research Instrumentats Ltd., Essex, UK) as per the manufacturers' instructions and quantified using the 3130XL Genetic Analyser and GeneMapper software (Applied Biosystems, Paisley, UK). Peak heights were taken as a measure of intensity.

MLPA data were analyzed using an adapted version of the Excel spreadsheet designed by Andrew Wallace, BSc, PhD, of the National Genetics Reference Laboratory, Manchester, United Kingdom (http://www.ngrl.org.uk/Manchester/), as described previously.[18] MLPA data obtained from frozen UM samples were normalized to MLPA data obtained from the frozen non-tumor control samples, while MLPA data obtained from FFPE uveal melanoma specimens were normalized to the data obtained from the FFPE non-tumor control samples.

The adapted analysis method allows for the removal of any control loci from an individual MLPA assay if the dosage quotient (DQ) is not within the range for a normal (disomic) copy number (0.85–1.15). MLPA data were considered reliable if the number of control loci within the normal range was ≥ 6 and if the SD of their DQs was <0.2. Of the three MLPA reactions performed for each sample, the reaction with the highest number of control loci (a minimum of 6) and the lowest standard deviation was considered the most reliable, and was used in all further analyses.

Chromosome copy number for 1p, 3p, 3q, 6p, 6q, and 8q was determined as loss (L), normal diploid (Di) or gain (G) only if $\geq$75% of the probes had DQs showing the following trends:

- Loss (L) = loss ($<$0.650) or borderline loss (0.650–0.849).
- Normal diploid (Di) = (0.850–1.150).
- Gain (G) = gain ($>$1.340) or borderline gain (1.151–1.340).

Where no consistent trend in $\geq$75% of the DQs was seen, the chromosome arm copy number was considered "unclassified" (U). Chromosome arm 8p was not included in the analyses due to the presence of only a single probe (*NRG1*) on this arm.

Results

Of the 20 paired UM samples included originally in this study, two FFPE samples failed to pass the minimum quality control criteria for MLPA, that is $\geq$6 control probes in the normal range (DQ 0.850–1.150) and a SD $<$0.2. Although the corresponding paired frozen samples did pass quality control criteria for MLPA, the two cases were excluded from all further analyses.

Patients

Clinical, histopathologic, and TNM Staging[35] data from the 18 UM cases included in this study are detailed in Table 1. The patients (10 female, 8 male) had a median age of 58 years (range 37–83). All tumors were choroidal, with three involving ciliary body and none demonstrated extraocular growth. The tumors had a median largest basal diameter of 16.7 mm (range 9.7–21.8). The treatment consisted of local resection in five patients and enucleation in 13, one of whom previously had received proton beam radiotherapy (S011). The melanoma cell type was spindle in eight tumors, epithelioid in one, and mixed cell type in nine. Closed extravascular matrix PAS positive closed loops were present in nine tumors. The median mitotic count was 6.5 in 40 HPF cases. By the close of the study in August 2011, four patients had had metastatic disease (Table 1).

Multiplex Ligation-Dependent Probe Amplification

Dosage quotients obtained for individual loci on chromosomes 1p, 3, 6, and 8 following MLPA analysis of FFPE and frozen tissue are detailed in supplementary Table 1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9584/-/DCSupplemental).

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Table 1. Clinicopathologic Features and Tumor Classification

				Clinical Features				Histopathologic Features				
	Tumor	**Sex**	**Age at PM (y)**	**Ciliary Body Involvement**	**Extraocular Extension**	**LBD (mm)**	**UH (mm)**	**Cell Type**	**Closed Loops**	**Mitotic Rate***	**TNM Stage†**	**Metastasis Present**
1	S164	F	58	N	N	9.7	3.2	Mx	N	<1	2a IIA	N
2	S165	M	48	N	N	16.8	9.5	Mx	Y	6	3a IIB	N
3	S167	F	67	N	N	14.3	16.8	S	N	5	4a IIIA	N
4	S168	M	81	Y	N	19.2	10.3	Mx	N	6	4b IIB	N
5	S169	F	46	N	N	17.6	9.6	S	Y	8	3a IIB	Y
6	S170	F	81	Y	N	16.6	10.2	Mx	Y	8	3b IIIA	N
7	S173	M	50	N	N	18.0	7.2	Mx	Y	16	3a IIB	N
8	S176	M	47	N	N	14.0	7.7	S	Y	15	3a IIB	N
9	S180	M	72	N	N	16.7	7.7	Mx	Y	7	3a IIB	N
10	S219	F	83	N	N	14.1	13.1	S	N	11	3a IIB	Y
11	S222	M	60	N	N	13.9	7.0	S	N	2	3a IIB	N
12	S007	F	43	N	N	14.4	10.2	S	N	2	3a IIB	N
13	S008	M	63	Y	N	21.8	12.2	E	Y	10	4b IIIB	Y
14	S009	M	77	N	N	19.5	14.9	S	N	8	4a IIIA	Y
15	S011	F	56	N	N	15.0	8.0	Mx	Y	4	3a IIB	N
16	S012	F	71	N	N	19.3	6.1	Mx	Y	14	4a IIIA	N
17	S013	F	37	N	N	12.4	10.6	S	N	5	3a IIB	N
18	S016	F	59	N	N	18.8	9.4	Mx	Y	4	4a IIIA	N

PM, primary management; LBD, largest basal tumor diameter; UH, ultrasound height; M, male; F, female; E, epithelioid cells; S, spindle cells; Mx, mixed cell type; N, no; Y, yes.

* Mitotic rate assessed as number of mitotic figures per 40 HPF.

† Based on the AJCC Cancer Staging Manual, 7th ed.[35]

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TABLE 2. Chromosome Arm Copy Number Detected by MLPA

	Tumor	Sample Type	Chromosome Arm					
			1p	**3p**	**3q**	**6p**	**6q**	**8q**
1	S164	Frozen	Di	L	L	Di	Di	G
		FFPE	U	L	L	Di	U	U
2	S165	Frozen	Di	L	L	Di	Di	G
		FFPE	U	L	L	Di	G	G
3	S167	Frozen	U	Di	Di	G	Di	G
		FFPE	U	Di	Di	G	Di	G
4	S168	Frozen	L	L	L	Di	Di	G
		FFPE	U	L	L	Di	Di	Di
5	S169	Frozen	L	L	L	U	Di	G
		FFPE	Di	L	L	U	Di	G
6	S170	Frozen	Di	L	L	Di	Di	Di
		FFPE	U	L	L	Di	Di	Di
7	S173	Frozen	Di	Di	U	G	L	G
		FFPE	Di	Di	U	G	L	G
8	S176	Frozen	L	L	L	Di	L	Di
		FFPE	L	L	L	L	L	U
9	S180	Frozen	L	L	L	Di	Di	Di
		FFPE	L	L	L	Di	Di	U
10	S219	Frozen	Di	Di	Di	Di	Di	Di
		FFPE	U	U	U	Di	Di	U
11	S222	Frozen	Di	Di	Di	G	G	Di
		FFPE	Di	U	Di	G	G	G
12	S007	Frozen	Di	Di	Di	Di	Di	Di
		FFPE	Di	U	Di	Di	G	Di
13	S008	Frozen	U	L	L	Di	L	G
		FFPE	L	L	L	L	L	G
14	S009	Frozen	L	L	L	Di	Di	G
		FFPE	L	L	L	L	Di	G
15	S011	Frozen	Di	Di	Di	Di	Di	Di
		FFPE	Di	Di	Di	U	Di	Di
16	S012	Frozen	Di	L	L	Di	Di	G
		FFPE	U	L	L	L	Di	G
17	S013	Frozen	Di	Di	Di	G	Di	Di
		FFPE	U	U	Di	G	G	U
18	S016	Frozen	Di	Di	Di	G	L	G
		FFPE	U	U	Di	G	L	G

Di, disomy.

The median number of control probes giving a disomic signal and, therefore, included in the normalization process, was 11.5 (range 8–12 probes) for the frozen UM samples and 7 (range 6–12 probes) for the FFPE UM samples. Table 2 summarizes the copy number for each chromosome arm, determined as described in Materials and Methods.

Chromosome arm 3p received the same classification in 13 cases of paired frozen and FFPE UM. For the remaining five UM cases, chromosome arm 3p was "unclassified" in the FFPE specimens, but their corresponding frozen tissue samples were classified as disomy when tested. For chromosome arm 3q, 17 paired frozen and FFPE UM samples received the same classification but one tumor showed an "unclassified" chromosome 3q copy number when the FFPE tissue was tested compared with a report of disomy 3 in the corresponding frozen UM tissue.

Copy number data for chromosome arm 8q showed 11 paired frozen and FFPE UM samples with concordant results. Of the seven paired frozen and FFPE UM samples with discordant results for 8q, five produced an "unclassified" result when the FFPE tissue was tested compared with either gain ($n = 1$) or disomy ($n = 4$) in the corresponding frozen UM sample. For the remaining two samples with discordant results for 8q, S168 showed a gain in the frozen UM sample but disomy in the FFPE sample with the converse occurring for UM case S122.

Chromosome arms 6p and 6q demonstrated copy number variations between paired FFPE and frozen UM samples as follows: five frozen UM samples were disomic for 6p but displayed either a loss ($n = 4$) or were unclassified ($n = 1$) in the corresponding FFPE sample; four frozen UM samples were disomic for 6q but displayed either a gain ($n = 3$) or were unclassified ($n = 1$) in the corresponding FFPE sample.

Chromosome arm 1p copy number was "unclassified" in eight FFPE UM samples, which were classified as disomy ($n = 7$) or loss ($n = 1$) when the corresponding frozen tissues were tested, and in one frozen UM sample, which was classified as a loss of 1p when the corresponding FFPE sample was tested. In addition, UM case S169 showed a loss of 1p in the frozen UM sample but was classified as disomy when examined in the corresponding FFPE tissue.

Discussion

To our knowledge, this is the first study to compare MLPA results for any malignancy from adjacent snap-frozen and fixed tissue samples from the same tumor. The sampling of adjacent

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areas of the tumor reduced the potential influence of tumor heterogeneity.

The semi-quantitative nature of MLPA is achieved by comparison of the peak heights for the test samples with control, non-tumor samples and control probes on chromosomes that are not perturbed by tumorigenesis. In the case of the P027.B1 UM assay applied in this study, control probes are directed to loci located on chromosomes other than 1, 3, 6, and 8. Therefore, to have a robust and reproducible assay, the results of control probes and control samples must be consistent between experiments, and also must have a DQ indicating disomy. The National Genetics Reference Laboratory, United Kingdom (NGRL) suggests that the control probes for each tumor should have an SD of less than 0.2 for MLPA results to be reliable (http://www.ngrl.org.uk/Manchester/publications). In 2010, our laboratory improved upon the suggestions of the NGRL by removing any control probes that did not have a DQ indicating disomy from the final analyses. That is, we consider tumor samples to be of acceptable quality only if they show six or more disomy control probes and a SD <0.2.[31]

Our data generated demonstrate that even where samples passed these quality thresholds, chromosome arm copy numbers were "unclassifiable" in some cases. For frozen UM tissue, this occurred in only a single chromosome arm in four of the 18 cases; 1p in S008 and S167, 6p in S169 and 3q in S173. For the fixed UM tissues, however, single or multiple chromosome arms were "unclassifiable" in all but two UM cases suggesting a low signal-to-noise ratio. This increased "noise" is most likely to affect DQs expected to be in the normal disomic range, since these values can be influenced in either a positive or negative manner, leading to an altered classification. Indeed, following MLPA testing individual chromosome arms gave "unclassifiable" results in FFPE material only when the corresponding frozen tissue produced a disomy result or was itself "unclassifiable."

Chromosome 3 copy number is important particularly when estimating the survival probability of patients with UM.[34] A normal disomic copy number indicates a good prognosis while the loss of one copy of chromosome 3 is a strong indicator of poor prognosis. We incorporate this information into an Accelerated Failure Time Model (AFT) together with clinical and histomorphological features of the UM to generate personalized prognostic curves, which also take into account the patient's age and sex.[34] Our data demonstrate that when we excluded FFPE UM tissue samples with either an "unclassifiable" chromosome 3p (28% of cases) or 3q (11% of cases) copy number, the remaining 12 FFPE samples showed 100% concordance with MLPA data from the corresponding frozen tissue, thus generating identical survival curves for these patients.

In a similar manner, when chromosome arms 1p, 6p, 6q, and 8q were examined individually, the exclusion of UM cases with an "unclassifiable" result in the FFPE sample resulted in the loss of 50%, 11%, 6%, and 28% of cases, respectively. The respective levels of concordance between the remaining paired frozen and FFPE UM samples for chromosome arm copy number were 88%, 75%, 82%, and 85%.

We showed previously that loss of a single locus on chromosome 3 correlates with reduced patient survival.[2] In the current study, the small cohort and the short patient follow-up do not allow meaningful evaluation of the prognostic significance of single locus deletions on chromosome 3 in fixed UM specimens. We hope to address this once we have sufficient data. Furthermore, a comparison of chromosomal abnormalities in irradiated tumors pre- and post-treatment would provide valuable information as to the genetic signature of aggressive UM cells that can survive such therapies.

In conclusion, when frozen tissue specimens of UM are not available for genetic analysis, FFPE tissue specimens can be used to determine chromosome 3 copy number, albeit accepting that some tumors will be unclassifiable.

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