Whole-Genome Microarray Detects Deletions and Loss of Heterozygosity of Chromosome 3 Occurring Exclusively in Metastasizing Uveal Melanoma

Sarah L. Lake,[1] *Sarah E. Coupland,*[1] *Azzam F. G. Taktak,*[2] *and Bertil E. Damato*[3]

Purpose. To detect deletions and loss of heterozygosity of chromosome 3 in a rare subset of fatal, disomy 3 uveal melanoma (UM), undetectable by fluorescence in situ hybridization (FISH).

Methods. Multiplex ligation-dependent probe amplification (MLPA) with the P027 UM assay was performed on formalin-fixed, paraffin-embedded (FFPE) whole tumor sections from 19 disomy 3 metastasizing UMs. Whole-genome microarray analyses using a single-nucleotide polymorphism microarray (aSNP) were performed on frozen tissue samples from four fatal disomy 3 metastasizing UMs and three disomy 3 tumors with >5 years' metastasis-free survival.

Results. Two metastasizing UMs that had been classified as disomy 3 by FISH analysis of a small tumor sample were found on MLPA analysis to show monosomy 3. No ubiquitous gene deletions of chromosome 3 were seen in the remaining 17 metastasizing disomy 3 UMs by MLPA. aSNP analysis revealed 95 deleted genes and 16 genes with loss of heterozygosity (LOH) on chromosome 3 in the disomy 3 metastasizing UMs that were not deleted or showing LOH in the nonmetastatic tumors.

Conclusions. MLPA can detect monosomy 3 cell populations in FFPE whole tumor sections previously missed by FISH performed on small tumor samples. Consistent deletion and LOH of genes on chromosome 3 occur in metastasizing disomy 3 UM and are detectable by aSNP analysis. Ninety-five genes were found to be deleted, and 16 genes showed LOH exclusively in disomy 3 metastasizing UM, suggesting a potential role for these genes in UM metastasis. (*Invest Ophthalmol Vis Sci.* 2010;51:4884–4891) DOI:10.1167/iovs.09-5083

Uveal melanoma (UM), the most common primary intraocular cancer in adults, is fatal in almost 50% of patients, because of metastatic spread often involving the liver. Chemotherapy of metastases has limited success[1,2] and disseminated disease is fatal in 92% of patients within 2 years of diagnosis. Clinical and histopathologic risk factors for UM metastasis include large basal tumor diameter (LBD), ciliary body involvement, epithelioid cytomorphology, extracellular matrix periodic acid-Schiff-positive (PAS^+) loops, and high mitotic count.[3,4] Prescher et al.[5] showed that a nonrandom genetic change, monosomy 3, correlates strongly with metastatic death, and the correlation has since been confirmed by several groups.[3,6–10] Consequently, fluorescence in situ hybridization (FISH) detection of chromosome 3 using a centromeric probe became routine practice for UM prognostication; however, 5% to 20% of disomy 3 UM patients unexpectedly develop metastases.[11] Attempts have therefore been made to identify the minimal region(s) of deletion on chromosome 3.[12–15] Despite these studies, little progress has been made in defining the key regions and/or metastasis-suppressor genes (MSGs) involved in UM metastasis.

We hypothesize that disomy 3 UMs that metastasize do so by the same mechanisms as metastasizing monosomy 3 UMs. However, instead of loss of a single copy of chromosome 3 facilitating this process, specific genes are deleted on chromosome 3 that are essential to an early progression to metastasis, not commonly seen in disomy 3 UM. The purpose of our study was to identify key MSGs that are deleted exclusively in a rare subset of UMs that metastasized despite apparent disomy 3 on FISH testing. We investigated whether deletions of chromosome 3 could be detected using either multiplex ligation-dependent probe amplification (MLPA) or a single-nucleotide polymorphism microarray (aSNP; SNP 6.0; Affymetrix, Santa Clara, CA). Knowledge of such deletions on chromosome 3 may allow more accurate prognostication, increase understanding of the natural history of UM, and help identify aberrant cell signaling pathways that may be amenable to therapy.

Materials and Methods

Tumor Samples

Fresh primary UM samples were routinely obtained at the Royal Liverpool University Hospital between 2001 and 2007 and analyzed by FISH for chromosome 3 copy number. Of these UMs, formalin-fixed, paraffin-embedded (FFPE) tumor samples were available in our archive for 34 disomy 3 UMs that were known to have metastasized (Di3M-UM). Nineteen of these samples were selected for MLPA studies, as they provided sufficient extracted DNA (700 ng) for quality control PCR and analysis by MLPA in triplicate. Four snap-frozen Di3M-UM samples from patients with fatal metastasis within 5 years of diagnosis and samples from three disomy 3 surviving UM (Di3S-UM) patients with no detectable metastases after a minimum of 5 years since diagnosis were used for aSNP analysis. Personalized survival curves were generated for all three patients with disomy 3 nonmetastasizing UM using the Cox proportional hazards model. The model predicts survival up to 8 years after diagnosis and specifies 95% CI based on the following information: age at treatment, sex, ciliary body involvement, largest basal

From the [1]Pathology Department, School of Cancer Studies, University of Liverpool, Liverpool, United Kingdom; the [2]Department of Medical Physics and Clinical Engineering, and the [3]Liverpool Ocular Oncology Service, St Paul's Eye Clinic, Royal Liverpool University Hospital, Liverpool, United Kingdom.

Supported by Grant Number 1685 from Fight for Sight (SLL); the European Organisation for the Research and Treatment of Cancer, Melanoma Group; the National Health Service, UK (AFGT) and the National Commissioning Group of the National Health Service, UK (BED, SEC).

Submitted for publication December 16, 2009; revised March 24 and April 12, 2010; accepted April 12, 2010.

Disclosure: **S.L. Lake**, None; **S.E. Coupland**, None; **A.F.G. Taktak**, None; **B.E. Damato**, None

Corresponding author: Sarah L. Lake, Department of Pathology, School of Cancer Studies, University of Liverpool, 6th Floor Duncan Building, Daulby Street, Liverpool, L69 3GA, UK; s.l.lake@liv.ac.uk.

Investigative Ophthalmology & Visual Science, October 2010, Vol. 51, No. 10

tumor diameter, extraocular spread, epithelioid cell type, mitotic count, the presence of closed extracellular matrix PAS^+ loops and chromosome 3 status. The curves predict the likelihood of metastasis-free survival of these patients at 8 years after diagnosis as being 61% to 79% (Supplementary Figs. S1A–C, http://www.iovs.org/cgi/content/full/51/10/4884/DC1).

No tumors used in MLPA or aSNP analysis developed from a preexisting nevus. Informed consent was obtained from each patient, and research was performed according to the tenets of the Declaration of Helsinki.

DNA Extraction

After examination of hematoxylin and eosin (H&E)–stained FFPE sections, areas with greater than 90% tumor cells were microdissected from 20-μm sections of each UM. DNA extraction was performed as described[16] (modified DNeasy Blood and Tissue kit; Qiagen, Crawley, UK). In brief, tissues were lysed in proteinase K buffer (1.6–0.8 mg/mL proteinase K, 50 mM Tris [pH 8.5], 0.1 M NaCl, 1 mM EDTA [pH 8.0], 0.5% Tween-20, 0.5% NP40, and 20 mM dithiothreitol) for 16 hours at 56°C followed by a further 24 hours at 37°C. The extraction protocol (DNeasy; Qiagen) was modified to include two washes with AW1 buffer. DNA was eluted in 50 μL of AE buffer. DNA quantity and A260/280 ratio were assessed with a spectrophotometer (NanoDrop; Thermo-Fisher Scientific, Cambridge, UK).

DNA was extracted from snap-frozen UM tissues (DNeasy Blood and Tissue kit; Qiagen) and purified with linear polyacrylamide (GenElute-LPA; Sigma-Aldrich, Poole, UK). Both of these steps were completed according to the manufacturers' protocols.

Quality Control PCR

A multiplex PCR, adapted from the technique of van Dongen et al.[17] was performed to ensure sufficient DNA quality for analyses. The 25-μL reactions contained 1× high-performance buffer, 2 mM $MgCl_2$, 0.5% BSA (Sigma-Aldrich), 0.8 mM dNTP mix, 0.625 units of polymerase (ThermoStart; ABgene-Thermo-Fisher Scientific), 0.1 μM forward and reverse primers for RAG1, PLZF, and AF4 exon 11, 0.2 μM forward and reverse primers for AF4 exon 3 (Eurofins; MWG, Operon, UK), and 100 ng of DNA. Reactions were performed with a TC-412 thermal cycler (Techne, Staffordshire, UK). PCR products were visualized on 2% agarose gels stained with 1× SYBR DNA gel stain (SYBR Safe; Invitrogen, Paisley, UK), using a gel-imaging system (Bio Doc-It Imaging System; Ultra-Violet Products Ltd., Cambridge, UK).

Multiplex Ligation-Dependent Probe Amplification

MLPA was performed with a probe assay (SALSA P027 assay; MCR-Holland, Amsterdam, The Netherlands), as described in Damato et al.[6] In brief, 200 ng of nontumor tonsil control and UM DNAs from FFPE tissues were used in each assay. MLPA reactions were performed with a thermal cycler (G-Storm GS1; Gene Technologies Ltd., Essex, UK). Amplified fragments were detected with a genetic analyzer (model 3130; Applied Biosystems, Foster City, CA), and software (GeneMarker; SoftGenetics, State College, PA), to determine peak heights as a measure of intensity. Tumor samples were tested independently three times.

Loss or gain of a chromosome arm was determined only if all MLPA probes on that arm showed loss/equivocal loss or gain/equivocal gain.

Whole-Genome Microarray

DNA (500 ng) from snap-frozen tissues was supplied to the Molecular Biology Core Facility (Paterson Institute for Cancer Research, Manchester, UK). Whole genome, SNP microarray analyses were performed, according to the manufacturer's protocols (Gene-Chip System and SNP 6.0 chip; Affymetrix). Raw data were analyzed for copy number variation (CNV) and loss of heterozygosity (LOH) using the unpaired, genomic segmentation settings in a commercial genetic analysis program (Genomics Suite; Partek, St. Louis, MO). An LOH baseline was generated by using the HapMap (http://www.hapmap.org) set of samples as a nontumor reference. Integration of the LOH and CNV data allowed detection of uniparental disomy. Gene annotations were added, using the University of Southern California, Santa Cruz Genome Project reference sequence identifiers (http://genome.ucsc.edu/index.html).

Tumor samples were grouped for further study as follows: (1) disomy 3 tumors that were fatal within 5 years of diagnosis (Di3M) and (2) nonmetastasizing disomy 3 tumors with >5 years' follow-up (Di3S). Genomic aberrations that were common and distinct to each group were identified. Our data analysis focused on chromosome 3 in particular, because of its strong correlation with metastatic disease, but we also examined chromosomes 1, 6, and 8.

Results

Patients

The 19 Di3M patients whose UMs were analyzed by MLPA comprised 11 women and 8 men with a median age of 66 years (range, 33–81). Clinical and histopathologic features of all UMs included in the study are summarized in Table 1, including TNM stage.[18] These UM had a median LBD of 17.3 mm (range, 8.7–21.5) and were classified histologically as being of mixed cell type in 12 cases, spindle in 4, and epithelioid in 3, according to the modified Callendar classification.[19] Closed extracellular matrix PAS^+ loops were detected in 12 of the UM.[3] The mitotic count, determined on H&E sections,[3] ranged from 1 to 25 mitotic figures per 40 high-power fields (HPFs), with a median of 7 (Table 1).

For the aSNP studies, the Di3M tumors from two women and two men, with a median age of 68.5 years (range, 68–84). The four UMs had a median LBD of 18.4 mm (range, 8.7–22). Histologically, three tumors were composed predominantly of epithelioid cells, and the remaining one was of spindle cell type. The median H&E mitotic count was 21 mitotic figures per 40 HPFs (range, 15–61/40 HPF). Two tumors had closed extracellular matrix PAS^+ loops. Nonfatal Di3S samples were obtained from two women and one man, with a median age of 64 years (range, 62–85). The median LBD was 16.2 mm (range 10.1–22.5 mm). All three tumors were composed predominantly of spindle cells and one had closed extracellular matrix PAS+ loops. The median mitotic count determined on the H&E sections was four mitotic figures per 40 HPF (range 3–6/40; Table 1).

Multiplex Ligation-Dependent Probe Amplification

Chromosome arm copy numbers detected by MLPA for all 19 Di3M-UM are shown in Table 2. From these data, two UMs, which were apparently disomy 3 on FISH testing, were recategorized as monosomy 3 (Figs. 1C, 1D). One of these tumors was polysomic for 8q. A third tumor showed deletion of 11 of 13 loci on chromosome 3 (Fig. 1E) and was still classified as Di3M by FISH and MLPA, as not all the 13 loci tested by MLPA showed deletion. One of the remaining 17 Di3M-UMs was found to show loss of the long arm of chromosome 3. Loss of 6q occurred in 50% of the Di3M-UMs and was the commonest gross chromosomal abnormality detected.

The most frequent gene deletions on chromosome 3 in the 17 confirmed Di3M-UMs included part of the *FHIT* gene at 3p14.2 (8/17) and the *MME* locus at 3p25.1 (7/17). Examination of each locus tested by MLPA on 1p, 6, and 8 individually in all Di3M-UMs showed that two loci were frequently amplified on 6p and 3 loci on 8q. These were *FOXC1*, 6p25 (12/17), and *CDKN1A*, 6p21.2 (10/17) for chromosome 6. On chromosome 8, two probes are situated in the *MYC* gene (8q21.12) and showed amplifications in 12 of 17 (nucleotides 528-588) and 14 of 17 samples (nucleotides 1659-1713). *DDEF1*

Table 1. Clinicopathologic Features and Tumor Classification

			Clinical Features		Histopathologic Features				
Sample ID	Sex	Age at Primary Management (y)	Extraocular Extension	Ciliary Body Involvement	Cell Type	Closed Loops	Mitotic Rate*	LBD (mm)	TNM Stage†
Di3M 3	F	69	N	Y	E	N	15	19.7	T4a IIIA
Di3M 4	M	81	N	N	S	Y	2	8.7	T2a IIA
Di3M 6	M	52	Y	Y	E	Y	2	10.6	T3a IIB
Di3M 7	F	74	N	Y	M	Y	1	14.5	T3a IIB
Di3M 10	M	76	N	N	M	Y	11	20.5	T4a IIIA
Di3M 13	F	58	N	Y	M	Y	2	20.5	T4a IIIA
Di3M 14	F	42	N	Y	M	N	11	21.5	T4a IIIA
Di3M 15	F	81	N	Y	M	Y	5	19	T4a IIIA
Di3M 16	M	56	N	N	M	N	5	19.1	T4a IIIA
Di3M 18	F	76	N	N	S	Y	2	18.8	T4a IIIA
Di3M 20	M	63	N	N	S	N	8	14.9	T2a IIA
Di3M 23	M	83	N	N	E	Y	25	17.3	T3a IIB
Di3M 25	F	62	N	N	M	Y	6	15.5	T3a IIB
Di3M 26	F	66	Y	N	M	Y	18	18.1	T4a IIIA
Di3M 27	F	67	N	N	M	Y	23	16.1	T3a IIB
Di3M 28	F	76	N	Y	M	N	7	17.5	T3a IIB
Di3M 29	M	33	N	N	S	N	14	14.8	T3a IIB
Di3M 30	F	44	N	N	M	N	1	13	T3a IIB
Di3M 31	M	41	N	N	M	Y	11	17	T3a IIB
Di3M 32	M	68	N	Y	E	N	61	22	T4c IIIB
Di3M 34	F	68	Y	Y	S	Y	17	8.7	T1d IIA
Di3S 1	M	62	Y	N	S	N	3	16.2	T4a IIIA
Di3S 2	F	64	N	Y	S	Y	6	22.5	T4a IIIA
Di3S 3	F	85	N	N	S	N	3	10.1	T2a IIA

LBD, Largest basal tumor diameter; M, Male; F, Female; E, epithelioid cells; S, spindle cells; M, 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, Seventh Edition.[18]

(8q24.2) was amplified in 10 of 17 samples. A single Di3M-UM harbored no deletions on chromosome 3 detectable by MLPA. Six further Di3M-UMs had only one ($n = 4$) or four ($n = 2$) equivocal deletions on chromosome 3 detected by MLPA.

SNP Microarray Analysis

Deletion and LOH were observed in both fatal disomy 3 metastasizing UMs and nonmetastasizing disomy 3 UMs on chromosome 3; a greater number of genes showed deletion in the metastasizing UMs and were unique to this group. The number of genes shown to have amplifications or deletions (copy number variations, CNVs) and LOH in all UM samples for each group of disomy 3 UM (metastasizing and nonmetastasizing) are detailed in Table 3.

As the purpose of the study was to detect genes involved in metastasis, we compared disomy 3 tumors known to have been

Table 2. Chromosome Arm Copy Number Detected by MLPA

	Chromosome Arm Copy Number						
Sample ID	1p	3p	3q	6p	6q	8p	8q
Di3M 3	D	D	D	G	D	D	G
Di3M 4	D	D	D	D	D	G	D
Di3M 6	D	D	D	D	D	D	D
Di3M 7	D	D	L	D	D	D	G
Di3M 10	D	D	D	D	L	D	G
Di3M 13	D	D	D	D	D	D	D
Di3M 14	L	D	D	G	L	D	D
Di3M 15	D	D	D	D	L	D	D
Di3M 16	L	D	D	G	L	D	G
Di3M 18	L	D	D	G	L	D	D
Di3M 20	L	D	D	G	L	D	D
Di3M 23	L	L	L	D	L	L	D
Di3M 25	D	D	D	D	D	D	D
Di3M 26	D	D	D	G	D	L	G
Di3M 27	D	D	D	D	L	D	G
Di3M 28	D	L	L	D	D	D	G
Di3M 29	L	D	D	G	L	D	D
Di3M 30	D	D	D	G	D	D	D
Di3M 31	D	L	D	D	D	L	G

D, disomy; L, loss; G, gain.

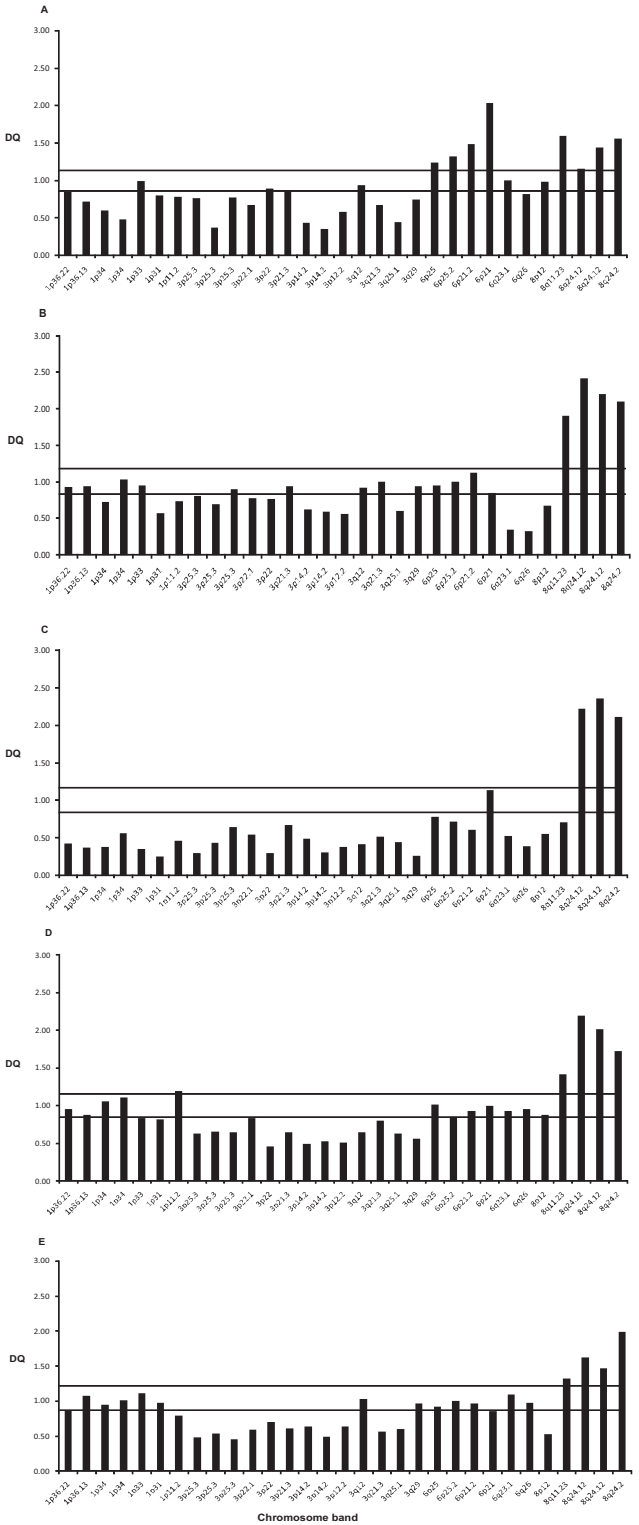

FIGURE 1. Graphs of MLPA dosage quotients (DQ). (**A**) Tumor Di3M 3 showing disomy chromosome 3 by MLPA, with unequivocal deletion of five probes on chromosome 3 at 3p25.3, 3p14.2, 3p12.2, and 3p25.1. (**B**) Tumor Di3M 27 showing disomy chromosome 3 and long arm 8 gain by MLPA with unequivocal deletion of three probes on chromosome 3 at 3p14.2, and 3p12.2. (**C**) Tumor Di3M 23, and (**D**) tumor Di3M 28 both showed monosomy 3 by MLPA, and Di3M 28 also showed gain of 8q. (**E**) Tumor Di3M 31 showed 11 of 13 loci on chromosome 3 deleted and 2 with normal chromosome copy number (3q29 and 3q12). DQs indicating normal chromosome copy number (disomy; DQ 0.85-1.15) fall between the two *horizontal lines*.

fatal with those with metastasis-free survival. The maximum number of years' follow-up for snap-frozen samples of disomy 3 UM with no metastasis in our archive was 6 years. Any locus found to be aberrant in both the Di3S- and Di3M-UMs was not thought to be essential for early metastasis within 5 years from diagnosis. CNVs on chromosome 1 consisted of a mixture of amplifications and deletions, whereas all CNVs on chromosome 3 were deletions. All chromosome 6 and 8 CNVs consisted of amplifications with the exception of the deleted *DEK* gene (6p22.3).

In total, 112 genes had deletions on chromosome 3: 95 genes specific to all Di3M-UMs (Table 4), 5 specific to all Di3S-UMs, and 2 common to both groups. Ten of the genes deleted on chromosome 3 in the Di3M-UMs also showed LOH in the Di3S-UMs. These genes were, therefore, not considered uniquely perturbed in the Di3M-UMs and are not included in Table 4, because LOH could have resulted in a similar loss of genetic information. Thirty-four genes showed LOH on chromosome 3: 16 specific to the Di3M-UMs (Table 5), 25 specific to the Di3S-UMs, and 3 common to all UMs analyzed. Of the 95 genes found to contain deletions on chromosome 3 in the Di3M-UM group exclusively, 3 showed LOH also (Table 3). Supplementary Tables S1–S4, http://www.iovs.org/cgi/content/full/51/10/4884/DC1, list the genes deleted or showing LOH exclusively in the Di3S-UMs and in all UMs analyzed.

Uniparental disomy (UPD) of chromosome 3 was not detected in any Di3M-UM. Aberrations on chromosomes 1, 6, and 8 occurring in all Di3M-UMs analyzed were: deletion of 227 genes and amplification of 1 gene on chromosome 6; deletion of 34 genes and amplification of 1 gene on chromosome 1; and amplification of 312 genes on chromosome 8. In the case of chromosome 8, 53 of the amplified genes also showed LOH. Five genes on other chromosomes were found to have CNVs only in Di3M-UMs: *PCBP3* (21q22.3), *PROKR2* (20p12.3), *PGR* (11q22.1), *DPP10* (2q14.1), and *TACR1* (2q13.1). A further 266 genes showed LOH across all the remaining autosomes.

DISCUSSION

Our investigation of this rare subset of Di3M-UMs demonstrates that: (1) MLPA of whole tumor sections can detect monosomy 3 in fatal UMs, whereas FISH of a small tumor sample could not; and (2) consistent deletion and LOH of multiple genes on chromosome 3 were detectable by aSNP exclusively in Di3M-UMs and therefore may indicate key UM-MSGs. These deletions and LOH events were undetectable by current FISH and MLPA assays used for UM prognostication.

To our knowledge, this is the most detailed study of Di3M-UMs yet conducted; however, the findings can only be tentative, because of the small number of tumors examined. The strengths of this study are the use of samples for which survival data were known, with more than 5 years' follow-up, and the use of a high-resolution technique capable of detecting CNV and LOH (aSNP). Our rationale of comparing CNV and LOH of genes in UMs known to have metastasized within 5 years of diagnosis and in UMs with no evidence of metastatic spread after more than 5 years postdiagnosis has enhanced the likelihood of detecting genes key to early metastasis. This analysis has, thereby, allowed us to remove those genes likely to be involved predominantly in UM initiation. The nonmetastasizing UM had a predicted 61% to 79% chance of metastasis-free survival 8 years after diagnosis. However, should samples have been available with longer metastasis-free survival, these would have improved our study by further removing genes involved in later metastatic progression.

aSNP was selected over methods detecting CNV alone (e.g., array comparative genomic hybridization) for two reasons.

Table 3. Number of Aberrations Detected by aSNP Analysis That Are Specific to Each Group of UM and Common to All UMs Analyzed

Sample Group	Class of Aberration	Chromosome			
		1	3	6	8
Di3M	CNV	35 (1)	95 (3)	228 (23)	312 (53)
	LOH	39	16	23	53
Di3S	CNV	26 (0)	5 (0)	336 (14)	2 (1)
	LOH	79	25	116	49
Common to all UM	CNV	10 (0)	2 (2)	34 (5)	35 (8)
	LOH	4	3	20	8

Data in parentheses denote the number of loci with LOH and a change in copy number for each group.

First, LOH is a common occurrence in malignancy and has been observed in UM[14]; therefore, it could be contributory to the metastatic phenotype of these tumors. Second, the combination of CNV and LOH data can detect the presence of uniparental disomy (UPD), reported to be present in 6% of UM.[20] The main weakness of this study is the small number of UMs tested, in particular using aSNP. This limitation is due to the rarity of metastasizing disomy 3 UMs and the paucity of snap-frozen frozen samples from these patients. However, in a small sample set, we have established that aSNP is the more appropriate method by which to detect deletions of chromosome 3 in future larger studies.

Chromosome 3 copy number detection by MLPA has already been shown to be superior to FISH when predicting patient survival.[6] In the present study, a direct comparison of MLPA and FISH was performed on a small subset of UMs and discrepancies between the two techniques observed. A significant finding of this study is that two Di3M-UMs were found to show monosomy 3 by MLPA. There are two possible explanations for this finding: (1) cellular heterogeneity in the sample, because of the presence of either differing tumor clones or of normal disomy 3 cell populations (e.g., macrophages or lymphocytes), or (2) greater sensitivity of MLPA than FISH. Consistent equivocal loss of chromosome 3 detected by MLPA has been shown to be clinically significant, suggesting increased sensitivity of MLPA over FISH.[6] However, the sensitivity of both FISH and MLPA will be influenced by the number of cells examined, the degree of cellular heterogeneity, and the individual cutoff values for detecting monosomy 3. Experiments to determine what contributes to variable MLPA results within UM are beyond the scope of this study, but are currently ongoing in our laboratory.[16]

MLPA can detect small deletions, as demonstrated by the observation of loss of one arm of chromosome 3 in the Di3M-UM. However, in the present study, no deletion of a single common gene was detected in all Di3M-UMs by MLPA. The most commonly deleted gene on chromosome 3 was *FHIT*, a member of the "fragile histidine triad" family, aberrant in many human cancers, and a putative tumor suppressor (reviewed by Pekarsky et al.).[21,22] Deletion of FHIT in fewer than half the samples tested here suggests that, although FHIT may play a role in tumor development, it is not a key MSG in UM.

In addition to monosomy 3, a strong correlation has been demonstrated between UM metastases and amplification of 8q.[6] Gain of the 8q gene *MYC* was the most frequent aberration of a single gene detected by MLPA, as previously observed in UM by FISH.[23,24] Two MLPA probes span the *MYC* gene and routinely showed different patterns of amplification. In this study, one locus was amplified in 14 UM and the other locus in 12. Further investigation of this finding is necessary to confirm whether a partial amplification is present in some UMs and its potential functional significance.

It is important to note that the 13 genes on chromosome 3 analyzed by MLPA were not specifically selected for their known involvement in UM. In addition, as only 13 loci were tested, large areas of chromosome 3 were not analyzed. This is demonstrated by the single UM for which no deletions of chromosome 3 were detectable by MLPA, and the 6 UMs for which only equivocal deletions were observed. Conversely, aSNP assays could detect multiple deletions of chromosome 3 in all samples tested. We conclude, therefore, that whereas MLPA with the P027 assay is superior to FISH in the detection of monosomy 3, in its current form it does not analyze the genes on chromosome 3 that may be key to UM metastasis in all patients. Should future studies determine deletions on chromosome 3 and aberrations of genes on other chromosomes that are essential to metastasis, the current MLPA assay could be adapted to include these and further improve UM molecular genetic prognostic testing.

By combining the LOH and CNV data from aSNP analysis, we assessed whether UPD was present in our cohort of Di3M-UMs as previously observed.[20] We hypothesized that UPD could be a cause of metastatic disomy 3 UM, with monosomy 3 tumor populations developing a disomy 3 phenotype, as a result of mitotic nondisjunction. However, none of the Di3M-UMs tested in this study, showed evidence of UPD. This finding may be a reflection of the small number of samples evaluated, and we are currently expanding these studies to include a larger cohort of patients.

UM is an unusual tumor, in that nonrandom gross chromosomal changes are frequently observed, the most common being monosomy 3, gain of 8q, loss of 1p, gain of 6p, and loss of 6q.[5,8,25,26] This observation was emphasized by the trends observed in the aSNP data, in which the majority of alterations occurred on chromosomes 1, 3, 6, and 8. We focused our analysis on these chromosomes because of their association with prognosis in UM, in particular monosomy 3. The 95 genes showing deletion and 16 genes showing LOH on chromosome 3, which occurred exclusively in all the Di3M-UMs analyzed, were regarded as the most likely to be key to early metastatic progression. The known function of these genes and potential involvement in UM or other tumors was investigated by using GeneCards (http://www.genecards.org/) and the published literature.

The most interesting aberrant genes on chromosome 3 were *RBM5*, *PPARg*, and *ROBO1*. *RBM5*, also known as *LUCA-15*, is a nuclear serine-rich related, RNA-binding protein involved in alternative splicing that has been shown to regulate apoptosis and proliferation. Downregulation of oncogenes by *RBM5*[27]

TABLE 4. Genes Showing Deletion on Chromosome 3 in All Di3M-UM Exclusively, Assessed by SNP Microarray

Cytoband	Gene	Cytoband	Gene
3p26.3	*CHL1*	3q13.11	*ALCAM*
	CNTN6	3q13.12	*IFT57*
3p26.2	*MDS1*	3q13.13	*BBX*
	LRRN1		*MYH15*
	SETMAR	3q13.2	*GTPBP8*
3p26.1	*GRM7*		*SLC9A10*
3p25.32	*RSRC1**		*CD200*
3p25.31	*KCNAB1*		*CD200R1*
3p25.2	*PPARγ*	3q13.3	*KIAA1524*
3p24.3	*OXNAD1*	3q13.31	*LSAMP*
	TBC1D5		*GAP43*
	KCNH8		*ZBTB20*
	ZNF385D	3q13.32	*IGSF11*
	DAZL	3q13.33	*GTF2E1*
	*UBE2E2**		*STXBP5L*
3p24.2	*THRB*		*NDUFB4*
3p24.1	*LRRC3B*		*HGD*
	AZI2	3q21.3	*KLHDC6*
	ZCWPW2	3q22.1	*CPNE4*
	RBMS3		*MRPL3*
	CMC1		*NEK11*
3p23	*GADL1*	3q23	*SR140*
	TRPC1		*CLSTN2*
3p22.3	*ARPP-21*	3q24	*SLC9A9*
	STAC		*PLSCR4**
3p22.2	*SCN10A*	3q24.3	*ANKRD28*
3p22.1	*MYRIP*	3q25.31	*TIPARP*
3p21.31	*CCR2*	3q25.32	*WDR49*
	DOCK3		*VEPH1*
	FLJ78302	3q25.33	*SCHIP1*
3p14.2	*CADPS*		*IQCJ*
	SYNPR	3q26.1	*SI*
3p14.1	*KBTBD8*		*SERPINI1*
	FAM19A1		*PPM1L*
	FAM19A4		*BCHE*
	SUCLG2	3q26.2	*SLC2A2*
3p12.3	*CNTN3*	3p26.3	*CNTN4*
	ZNF717	3q26.32	*KCNMB2*
	ROBO2	3q26.31	*TNIK*
	ROBO1		*SPATA16*
3p12.2	*GBE1*	3q26.33	*PEX5L*
3p12.1	*CADM2*	3q28	*TMEM207*
3p11.2	*EPHA3*		*LEPREL1*
3q11.2	*OR5K1*		*TPRG1*
	EPHA6		*FGF12*
	OR5K2		*IL1RAP*
3q12.1	*COL8A1*	3q29	*ATP13A5*
3q12.2	*ABI3BP*		

* Genes showing LOH also.

and inhibition of lung cancer cell line growth[28] have led to the implication that *RBM5* is a tumor-suppressor gene.

ROBO1 is a member of the immunoglobulin gene superfamily and encodes a membrane receptor protein that functions in axon guidance and neuronal precursor cell migration after activation by SLIT family proteins. It has been described in many different cancers, but the literature conflicts with respect to its role in carcinogenesis. Studies in head and neck, breast, and clear-cell renal carcinoma have shown that *ROBO1* downregulation[29,30] is associated with poor patient prognosis.[31] Conversely, in hepatocellular and colorectal carcinoma, *ROBO1* is highly expressed.[32,33] The former finding supports our results that *ROBO1* is deleted in metastasizing UM. Moreover, a recent study demonstrated, in the melanoma cell line MDA-MB-435, that an active Slit/Robo signaling pathway limits cellular migration, causing the cells to remain confined within their primary location.[34]

PPARg (peroxisome proliferator-receptor gamma) had also been implicated in many cancers. Decreased expression is associated with poor prognostic indicators in breast cancer,[35] prostate cancer,[36] Burkitt's lymphoma,[37] and colorectal cancer.[38] More interesting, however, is the interaction of PPARγ with β-catenin.[35] β-Catenin is an integral part of the Wnt signaling pathway, with decreased levels of both proteins being associated with liver metastasis in colorectal tumors.[39] Studies in cutaneous melanoma show PPARγ agonists to have antiproliferative effects and modulate Wnt/β-catenin signaling.[40] In UM, increased β-catenin and Wnt5a expression are associated with poor survival.[41] Further investigation of the expression levels of these proteins in a panel of metastatic and nonmetastatic UMs, as well as their influence on the migratory properties of UM cell lines, are necessary to further delineate their importance in metastatic UM.

The strong correlation of 8q amplifications with poor prognosis in UM patients suggests that genes promoting UM metastasis may also be located elsewhere in the genome. The 312 genes amplified on chromosome 8, exclusively in all the Di3M-UMs analyzed, were on 8q; 53 of these genes also showed LOH. Thus, it is possible that their gain was not advantageous to metastatic progression, and therefore LOH may have been favored during UM development. Among the genes showing amplification but no LOH on 8q were *MYC* and *DDEF1*. *MYC* amplification has been observed in UM by FISH, but its role in tumor metastasis is still unclear, even though it is also amplified in liver metastases of UM.[23] Moreover, early studies unexpectedly noted an association between *MYC* expression and good patient prognosis.[24,42,43] Elevated levels of DDEF1 have been detected in UM, and ectopic expression of this factor increases motility in low-grade UM cell lines,[44] suggesting a role in metastatic progression. It is unlikely that a single gene controls UM metastasis, and therefore it is important to consider all likely candidates together, despite their chromosomal location, to determine how they may interact to promote tumor progression.

Previous studies attempting to define the minimal regions of loss on chromosome 3 in UM have implicated 3p25-pter,[45] 3p25–6 and 3p11–14,[12] 3p25.1–25.2,[13] 3p25 and 3q24–26,[15] and 3p13[46] as potentially harboring tumor-suppressor genes. Although it was not the intention of this study to detect a minimal region of loss, it is interesting to note that some of the genes detected as exclusively deleted in all fatal Di3M-UMs, including *PPARg* and *ROBO1*, do fall within these regions.

In summary, our investigation of this precious cohort of Di3M-UMs has demonstrated both gene deletions and LOH on

TABLE 5. Genes Showing LOH in All Di3M Samples Exclusively, Assessed by SNP Microarray

Cytoband	Gene
3p35.32	*RSRC1*
3p25.1	*SLC6A6*
	SERP1
	EIF2A
3p24.3	*UBE2E2*
3p24	*PLSCR4*
3p22.1	*MOBP*
3p21.31	*RBM5*
	RBM6
	GNAT1
	GNAI2
	SEMA3F
3p21.3	*SLC38A3*
3p13.13	*PVRL3*
3p11.2	*HTR1F*
3q21.3	*TRH*

chromosome 3, which were undetectable by current FISH and MLPA assays used for UM prognostication. Because the number of tumors examined was small, the gene aberrations detected in this study of Di3M-UM are preliminary and require validation in a larger cohort of UM. This research will be performed in the near future by our group and will allow the refinement of the list of aberrant genes on chromosome 3 and the long arm of 8 that occur solely in metastatic UM. Inclusion of monosomy 3 metastatic UM in any larger cohort of samples studied may identify homozygous deletions of potential MSGs and would also help confirm whether these two genetic subtypes of UM develop their propensity for metastasis via the amplification of a similar subset of metastasis promotion genes on 8q. Our future work will focus on a "systems-biology" approach to prioritize those genes likely to be master regulators of metastasis, followed by functional assays to confirm their influence on metastatic characteristics such as cellular invasion, motility, and plasticity.

Acknowledgments

The authors thank Gillian Newton and Stuart Pepper (Paterson Institute of Cancer Research) and Mike Churchman (St. James's University Hospital, Leeds) for their technical expertise and guidance in performing genomic microarrays.

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