High-Resolution Array CGH Analysis Identifies Regional Deletions and Amplifications of Chromosome 8 in Uveal Melanoma

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Purpose. Monosomy 3 (M3) and abnormalities of chromosome 8 associate with poor prognosis in uveal melanomas (UM). Although M3 has been the subject of more in-depth studies, none have intensively focused on chromosome 8. To elucidate the potential role of chromosome 8 abnormalities, array comparative genomic hybridization (aCGH) was performed on primary UM.

Methods. A specifically-designed custom high-resolution array was developed focusing on changes most implicated in UM. Probes for chromosome 8 had a mean spacing of 2.3 kb while chromosomes infrequently affected had a mean spacing of 36.6 kb. A series of 75 UM, including one formalin-fixed paraffin sample were analyzed, and where possible control DNA extracted from the patient's own peripheral blood was used.

Results. The most common copy number abnormalities were chromosome 8 (75%) and M3 (51%), with M3 and gain of the long arm of chromosome 8 (8q+) associated in 41% of cases. Also identified were partial deletions of chromosome 3 (3%) and regional 8q+ (23%), and the intensive coverage of chromosome 8 revealed small focal deletions and amplifications affecting both arms. The most significant predictor of prognosis was M3/8q+ having a hazard ratio of 10.1 ($P < 0.0001$).

Conclusions. Neither 8p deletion nor focal changes affecting chromosome 8 were linked to outcome. The most significant indicator was M3/8q, and multiple 8q+ associated with shorter survival. Studying UM with this technology provides a powerful robust tool for predicting prognosis while considering other genetic changes, allowing the future incorporation of such data as it becomes clinically significant.

Keywords: melanoma, genetics, chromosome 8, uveal

Uveal melanoma (UM) is the most common primary intraocular tumor of adults, with a highly aggressive nature. Approximately 50% of affected patients die from their disease.[1] The survival of patients with UM is almost entirely dependent on whether they develop liver metastases because once these are detected the prognosis is extremely poor (averaging 6 months), reflecting the multifocal and highly chemo-resistant nature of these lesions, which renders them inoperable and unresponsive to current therapies.[2] New therapies such as the use of mitogen-activated protein kinase kinase (MEK) inhibitors may however prove of value in the future and the area is the subject of intense research.[3] Approximately 500 new cases are diagnosed in the United Kingdom each year and Sheffield, as a national center for the treatment of UM, sees approximately 150 cases annually.

Previous research has shown that UM are characterized by changes of chromosomes 1, 3, 6, and 8, and a relationship has been identified between abnormalities of chromosomes 3 and 8 and a poor prognosis.[4–8] Changes of chromosome 1 also relate to poor prognosis, while those of chromosomes 6 and 11 indicate patients with a better outcome or associate with a less aggressive cell type.[4,5,8] Although loss of a complete copy of chromosome 3 (monosomy or M3), detected by a variety of methods, has long been associated with a worse prognosis in UM, this characteristic alone has not always proved sufficient in predicting which patients are likely to develop metastases. More recently, there has been speculation as to the genes involved in metastatic progression, and although the *BAP1* gene is potentially targeted through M3 and is an interesting candidate,[9,10] little is still known about the genetic drivers of metastasis in UM. Evidence however suggests that changes of chromosome 8 are subsequent to M3 and may therefore facilitate the process.[11,12]

An additional gain of 8q, particularly with the concomitant loss of 8p, has proved to be a good predictor of poor prognosis, specifically when considered in combination with M3.[5,12,13] The incidence of 8q gain ranges from 55% to 75% of primary UM depending on the technique used.[14–18] The entire q arm is often amplified and cytogenetic studies initially defined a

minimal regional of gain as 8q21-qter,[19] which was subsequently refined by spectral karyotyping and comparative genomic hybridization (CGH) to suggest that two distinct regions of 8q may be amplified, at 8q21.1-21.2 and 8q23-24.[16,18,20,21] Genetic studies of the metastases of UM have suggested gain of 8q may be particularly related to their development, since 8q+ is a consistent finding, whereas other changes, such as the loss of chromosome 3, are not always present. In earlier reports of 16 metastatic lesions of UM, gain of all or part of 8q was found in all but one instance, while abnormalities of chromosome 3 were less common and found in only 10 of 16 cases.[6,22–24] Furthermore, in a recent bacterial artificial chromosome (BAC)-based array CGH study, gains of 8q, often at a high level, were the most common abnormality found in the 66 UM liver metastases studied, 89% compared with M3 in 73%.[12] It is also important to consider the manner in which the gain of 8q arises, mostly in the form of an isochromosome and in a recent single nucleotide polymorphism (SNP)–based study, 8p loss (and M3) remained an independent predictor of poor metastatic outcome after adjusting for the effects of all other variables.[25]

Besides being the most frequent observation amongst metastatic UM lesions, evidence suggests that the greater the number of copies of amplified 8q, the shorter the disease-free interval,[5] and in a recent gene expression study, large regions of 8q were shown to be overexpressed in those cases with a short disease free survival.[26] Taken together, these findings suggest that genes targeted by gain of 8q are important to the progression and development of metastases by UM.

Uveal melanomas have been studied previously by array CGH, but at a relatively low resolution of up to 40 kb median probe spacing.[12,13,21] In this context, we have designed a high-resolution CGH array in order to specifically investigate abnormalities of chromosome 8, while also covering other abnormalities related to UM at a higher resolution.

Materials and Methods

Clinical Samples

The series comprises 75 primary UM from patients treated at the Royal Hallamshire Hospital (Sheffield, UK) during the period 1994 to 2014. All patients underwent enucleation. Immediately following the procedure in theater, samples were placed in cryovials, collected into liquid nitrogen and stored at −80°C until required. Informed consent was obtained from all UM patients with ethical approval (SSREC 94/247 and 09/H1008/141) and the procedures adhered to the tenets of the Declaration of Helsinki. One sample was obtained from archival formalin-fixed paraffin (FFPE) material. Within the series, approximately one-half the patients had a long follow up/known outcome in combination with cytogenetic analysis. The remainder were analyzed either prospectively or selected on the basis that good quality frozen samples were available.

In the series as a whole, 43 patients were male and 32 were female. There were 44 cases from the choroid, 10 from the ciliary body and 21 from a combined location. Forty were of mixed cell type, 27 were spindle cell, and 8 epithelioid. Twenty-six cases had developed distant metastases. The average median tumor diameter was 14.7 mm (smallest 6.3, largest 22.2). This data is available in Supplementary Table S1.

The mean age at surgery was 61 years, with the youngest aged 22 and the oldest 87. The mean survival following surgery was 48 months (shortest 2, longest 201).

CGH Microarray

Design. A CGH microarray was designed using the eArray tool from Agilent (Santa Clara, CA, USA). For each chromosome, probes were selected to ensure an even coverage. Different densities of probes were chosen, so that those chromosomes previously identified as significant in uveal melanoma were covered with a greater number of probes.

For chromosome 8, the mean interprobe spacing was 2.3 kb, 14.5 kb for chromosomes 1, 3, 6 and 11, and 36.6 kb for the remainder, apart from Y, where the density was approximately one probe every 1443.5 kb. A comparable "off the shelf" design from Agilent (e.g., G4449A has a 13-kb overall median probe spacing [11 kb in *RefSeq* genes]).

An additional 11,539 probes were included as controls and replicates by the manufacturer and the arrays were provided in a 4 × 180 k format.

Sample Processing and Microarray Procedure. DNA was extracted from snap frozen UM tumor samples using the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK). The FFPE sample was treated as previously described.[27] DNA concentration and quality was measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reference DNA was obtained from peripheral blood samples for each patient. Blood samples were not available in one case and commercial DNA (Promega UK, Southampton, UK) was used as a reference.

One microgram each of test and reference DNA was double-digested with Alu I and Rsa I then labeled separately with Cy5 and Cy3, respectively, with a labeling kit (Agilent), according to the manufacturer's instructions. The FFPE sample was labeled using a universal linkage system (ULS) technique as previously described.[27] After cleanup of labeled DNA, the yield and specific activity of each sample were determined using the NanoDrop. Test and reference DNA were combined and coprecipitated with Cot1 DNA. Arrays were hybridized for 24 hours (30 hours for the FFPE sample) at 65°C in the rotator rack of a hybridization oven set at 20 rpm. After washing, arrays were scanned with an Agilent G2505C scanner at a resolution of 3 μm.

Analysis. Data was extracted with Feature Extraction 10.1 and then analyzed using the ADM2 algorithm from the Genomic Workbench analysis package (Agilent). The Common Aberration algorithm from the same package was used to assist in the identification of abnormal regions of chromosome 8 present in more than one case.

Statistical Analysis

Analysis was carried out using SPSS Statistics v21 (IBM UK, Portsmouth, UK). The survival distributions of cases with abnormalities of chromosomes 3 or 8 were estimated by the Kaplan Meier method. Other characteristics of the tumor series were compared for their contribution to survival using the Cox regression model.

Results

Seventy-four of 75 cases showed copy number changes, with considerable variation in the levels of amplification found at different chromosomal loci. In most cases, DNA from the patient's own circulating lymphocytes was used as the reference. This offered the considerable advantage of controlling for copy number variation, ensuring that any copy number changes found were likely to be accurate and genuinely associated with the disease. The array had been designed specifically with UM in mind and was concentrated on known associated chromosome changes (1, 3, 6, 8, and 11). Although a wealth of data exists on the relevance of these changes, the most consistently linked with prognosis are those of 3 and 8, which often occur together.

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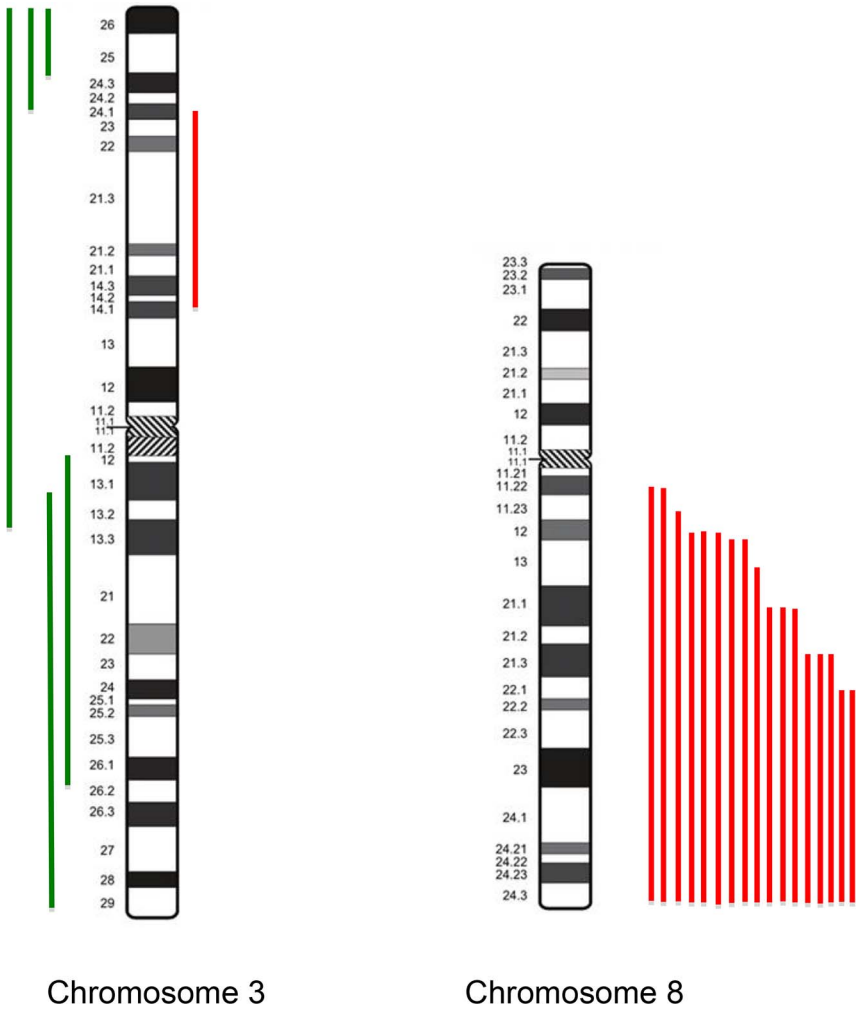

FIGURE 1. Range of partial losses (*green*) and gains (*red*) of chromosome 3 (4 cases) and chromosome 8q (17 cases) in uveal melanoma. Partial 8q gains were associated with partial deletions of chromosome 3 in 4 of the 17 UM, and in one instance, partial 8q gain was associated with both regional losses and gain of chromosome 3.

In this series the frequency of changes affecting the most commonly altered chromosomes was as follows 1p-/q+ (40%), M3 (51%), 6p+ (40%), 8q+ (75%), 8p− (28%). The most frequent alteration 8q+, affecting 56 cases (75%), comprised UM with whole-arm gains and also those with only a partial gain of material on 8q. Monosomy 3 was found in 38 cases (51%) and in most instances was associated with a gain of at least one copy of 8q (31 cases). In only one UM was a partial gain of 8q associated with M3 and the most common relationship was between 8p loss and UM cases with both M3 and 8q gain, corresponding to the previously observed close association between M3 and isochromosome 8.[5,28]

Breaking this series into those cases with entire or partial gains of 8q the largest group, 39 cases (52%), had gained at least one whole extra copy of 8q, and in nine cases this corresponded to gain of an entire chromosome 8 (simultaneous gain of 8p and 8q). For the remaining cases where only 8q was gained, approximately one-half had multiple gains, with nine cases having two extra whole copies, while five cases had three or more. The gain of 8q was accompanied by a loss of 8p in 21 cases (28%), but no losses of 8p were found without a concomitant whole gain of 8q. For the second group, cases with only partial gain of 8q (17 cases, 23%), there was a considerable range of partial gains affecting the long arm (Fig. 1). Furthermore all cases with regional loss of chromosome 3 were associated with partial 8q gain. In one instance, this involved both regional loss and gain of chromosome 3 (loss from 3pter to 3p24.1 and a contiguous gain from 3p24.1 to 3p14.1). Abnormalities of chromosome 6 (38 cases, 51%) mainly involved the gain of material from 6p (40%) and 30 cases (40%) had abnormalities of chromosome 1, of which the overwhelming majority showed loss of material from the short arm. There was general agreement between the results of aCGH and previous cytogenetic analysis (see Supplementary Table S1).

In univariate analysis, only cell type and genetic status were identified as significant variables. Kaplan Meier survival curves for these variables are shown in Figure 2.

The hazard ratios associated with different genomic abnormalities are shown in Table 1.

Adding various tumor characteristics such as age at operation and sex of the patient into a Cox proportional hazards model of survival gave only genetic status as being significant. The combination of M3 and gain of at least one whole copy of chromosome 8q proved the most powerful, with a hazard ratio of 10.1 ($P < 0.0001$). Overall, shorter survival and risk of metastasis appears to be predicated by the presence of M3 accompanied by at least one additional copy of 8q. Using these two abnormalities together as a "test" for metastatic potential had a sensitivity of 81% and a specificity of

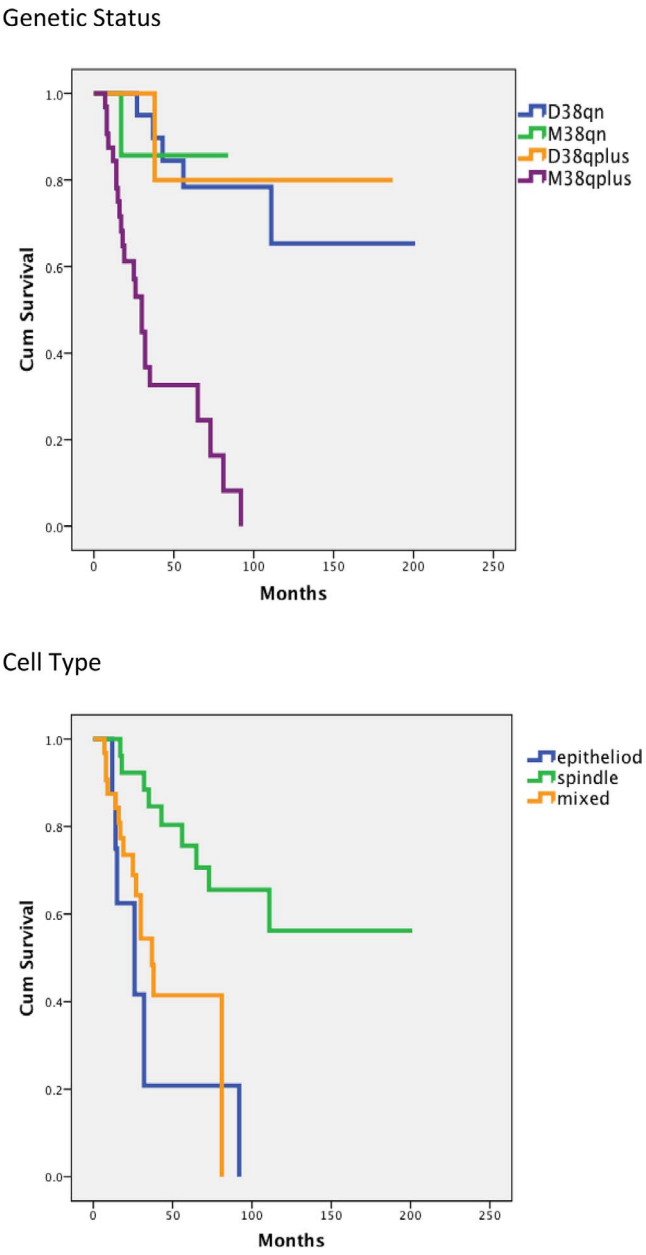

Figure 2. Kaplan Meier survival curves for Genetic Status of chromosomes 3 and 8 (*upper graph*) and Cell Type (*lower graph*) in 75 UM. D3, disomy 3; M3, monosomy 3; 8qn, 2 copies of 8q; 8qplus, additional copies of 8q.

Table 1. Hazard Ratio and Significance of Genetic Aberrations in a Series of 75 Cases of Uveal Melanoma

Aberration	Hazard Ratio	95% CI	*P* Value
M3/8q+	10.105	3.431–29.762	<0.0001
M3	8.904	3.262–24.305	<0.0001
8q+	5.828	2.334–14.555	<0.0001
8p−	5.671	2.674–12.027	<0.0001
Partial 8q+	0.474	0.177–1.267	0.109
8p+	0.555	0.168–1.835	0.335
1p−	1.186	0.563–2.495	0.654
6p+	0.254	0.106–0.609	0.001

Table 2. Hazard Ratios of Differing Copy Number Gains of 8q in 75 UM

Aberration	Hazard Ratio	95% CI	*P* Value
Gain of 1 copy of 8q	5.557	2.105–14.667	0.001
Gain of 2 or more copies of 8q	6.306	2.222–17.897	0.001

80%. Employing either M3 or 8q+ alone gave a better sensitivity (89% and 85%, respectively) but a much worse specificity (68% and 64%). It is difficult to determine if partial 3 loss and associated partial 8q gain have accordingly higher risk as only four cases were identified, and although one patient had died another was alive at 111 months, with average follow up of 40 months, ranging from 4 to 111 months. Furthermore, in this cohort 16 of the 39 cases with 8q+ (21% of the series) had more than one extra copy of 8q. These latter cases had an overall mean survival of 31.9 months, compared with 41.1 months for those with only a single gain of 8q. Hazard ratios for differing copy numbers of 8q are shown in Table 2, supporting the suggestion that 8q copy number has prognostic implications, and increased gain correlates with a reduced disease free interval following initial diagnosis.

Paying particular attention to alterations affecting chromosome 8, we observed that, in addition to whole arm–based abnormalities, a number of small focal amplifications and deletions were identified. On 8p, these focal events occurred at a number of recurring loci, whereas on 8q they were fewer and appeared to be widely scattered. The occurrence of focal imbalances in relation to other changes of chromosomes 3 and 8 is detailed in Table 3.

Focal amplifications were observed in 21 cases (28% of series), and 19 cases had focal deletions of 8p (25% of series). The number of cases affected by specific regions of focal deletion and the genes within are detailed in Table 4. Only one region was subjected to a higher incidence of focal amplification and is also detailed in Table 4. The most commonly implicated region for both deletion and amplification was 8p21.3 with seven cases showing a defined focal deletion and interestingly 12 cases having focal amplifications. Of those cases with focal deletions, nine UM had more than one focal deletion and seven UM had both focal amplifications and deletions of 8p, although not affecting the same regions. One of these cases had both an amplification and deletion of 8p21.3, but affecting different regions, suggesting hypervariability in the region. No associations were found between survival, cell type, tumor location, or age with any of the loci identified and the focal abnormalities did not correlate specifically with other genetic imbalances. For example, the

Table 3. Association of 8p Imbalances with M3 and 8q in 75 Cases of UM

	8q Normal	8q Gain Only	8q Gain, M3	8q Partial Gain	M3 Only	8q (fa/fd)
8p normal	3	0	0	6	0	4
8p gain	0	6	3	0	0	0
8p loss	0	1	20	0	0	0
8p (fa)	3	0	6	7	1	4
8p (fd)	2	0	6	6	0	5

The status of 8p, including focal amplifications and deletions, is compared with the relationship to 8q and M3 in a series of UM. Note: Some UM cases had both focal amplifications and deletions and are therefore represented on more than one occasion. M3, monosomy of chromosome 3; gain/loss, whole arm changes; fa, focal amplifications; fd, focal deletions.

Table 4. Regions of Focal Deletion or Amplification of 8p Found in 21 Cases of UM

Locus	Number of Cases	Genes in Region	Smallest Common Deletion Found
8p11.21 (del)	5	*MYST3 AP3M2 PLAT VDAC3 DKK4 POLB IKBKB*	480 kb
8p12* (del)	11	*GSR UBXN8 PPP2CB*	100 kb
		FUT10 AK308918 MAK16 C8orf41 RNF122 DUSP26	325 kb
		GPR124 BRF2 RAB11 FIP1 GOT1L1 ADRB3 EIF4EBP1 ASH2L STAR LSM1 BAG4	420 kb
8p21.3 (del)	7	*PIWIL2 SLC391A4 PPP3CC*	65 kb
8p21.3 (amp)	12	*EGR3*	3 kb
8p23.1 (del)	6	*MFHAS1 ERI1*	125 kb

Loci that were implicated in five or more cases are shown. Genes deleted at each locus are listed in the table.

* Three discrete regions of deletion were found at 8p12. Some cases are represented on more than one occasion, having more than one focal change.

most frequent focal amplification of 8p affected 8p21.3 and specifically targeted the *EGR3* as the sole gene at this locus in 12 cases, but was found in UM with otherwise deleted 8p, and those with no deletions of 8p.

Discussion

In this study, changes in chromosome 8 were the most common copy number abnormality. A wide range of techniques has been used to investigate the genetics of UM, including cytogenetics, fluorescence in situ hybridization (FISH), SNP analysis, multiplex ligation-dependent probe amplification (MLPA), and gene expression analysis. These have all associated loss of M3 and often gain of the long arm of chromosome 8 with a worse outcome in UM, albeit with a considerable range of predictive success, and recently studies have identified 8p loss as correlated with metastasis.[25] Copy number abnormalities of 8p were common in this series (41% of cases), although never occurring without an accompanying change of 8q, and the data gave no indication that these changes were independently related to patient prognosis. Extra copies of the long arm of chromosome 8 have been previously associated with both metastatic spread and with a shorter disease free interval in UM[4–8] and this study supports these earlier observations. Furthermore, an association between increased copy number of 8q and worse prognosis was previously established using whole-arm data derived from cytogenetic or FISH studies,[5,29] and the evidence from this investigation supports this association. There is, however, little information available on partial gain of 8q in UM and the degree to which this is associated with outcome.

The CGH arrays used in these experiments were specifically designed to assess chromosome 8 for copy number changes at a higher resolution than had been previously employed.[12,13,21] This study indicated that there was a range of sizes for the regions partially gained on 8q (Fig. 1). Here the findings suggest that the minimal region of gain is 8q22.1-8qter, which is in agreement with previous suggestions on the size of minimal deletion.[16,18,20,21] These cytogenetic and early CGH studies cannot provide evidence to suggest anything other than that the region is gained uniformly with consistent amplification throughout and the same increased copy number. More recent investigations at higher resolution have not undertaken such a focused examination of individual regional variations. We specifically chose to focus on the abnormalities affecting chromosome 8 and the high-resolution analysis performed in this investigation; using the custom array developed specifically for UM shows, however, that these partial gains are not equally amplified, whereas for the UM that gain an entire copy of 8q, it was found there was consistent amplification throughout.

It is also of interest that this study identified small focal deletions of 8p at four separate loci with some of these loci (e.g., 8p21 and 8p23) having been frequently associated with a number of other malignancies,[30,31] although no definitive driver genes have as yet been identified. Most of the genes affected by these focal changes have potential roles that could implicate them in the development and progression of UM. More specifically we found a focal amplification of 8p, targeting the *EGR3* gene in 12 UM. Although *EGR3* overexpression has been associated with malignancy (e.g., in prostate cancer[32]), it is difficult to conclude a definitive role for the amplification of this gene in UM, as it shows no correlation with other known indicators of disease progression. Specifically, for example, the amplification of the *EGR3* gene was found amongst UM with M3 and no M3, 8q+ and no 8q+. In terms of 8p itself, the focal gain of *EGR3* was present even in UM with no imbalance of 8p, or where the rest of the arm was deleted. Likewise, other focal abnormalities did not appear to group with any other genetic change and none were associated with the likelihood of developing metastatic disease. As the number of cases with these changes is small it is difficult to draw conclusions on their relevance as potential drivers of disease progression in UM. As matched control DNA from the patients themselves was used it is unlikely that copy number variation (CNV) would be responsible for these observations, or that they are artifactual since 8q+ was not subject to comparable focal variation. The findings suggest that there is much more hypervariability in UM than previous studies using lower resolution have suggested.

The evidence from this investigation shows despite the overall appearance of global integrity found in earlier studies, there is much flexibility in the size of amplifications of 8q gain as well as the focal nature of changes affecting 8p. Uveal melanoma have recently been suggested as having a low level of mutational events in targeted cancers genes[33] and through larger scale sequencing shown to be relatively genetically stable.[34] Recent studies of cutaneous melanomas demonstrated that they can have a crisis of focal instability related to a particularly poor outcome, termed chromothripsis.[35] It is unclear if a similar situation exists here in UM. This high-intensity study, focusing particularly on chromosome 8 however reveals there is a previously undetected high level of instability affecting chromosome 8 and that the genetic landscape of UM still has many secrets to reveal, being not as flat as was initially thought.

In summary, studying UM with this array gives a powerful and consistent way of assessing outcome for these patients. As investigations continue into the genetic factors influencing progression and outcome in UM, aCGH is likely to prove a powerful tool for both research and more detailed prognostication.

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