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Authors
Hughes, S
Damato, BE
Giddings, I
et al.

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Microarray comparative genomic hybridisation analysis of intraocular uveal melanomas identifies distinctive imbalances associated with loss of chromosome 3

S Hughes1, BE Damato2, I Giddings3, PS Hiscott2, J Humphreys3 and RS Houlston*,1

1Section of Cancer Genetics, Institute of Cancer Research, Sutton, SM2 5NG, UK; 2Liverpool Ocular Oncology Center, Royal Liverpool University Hospital, Liverpool L7 8XP, UK; 3Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton SM2 5NG, UK

Defining regions of genomic imbalance can identify genes involved in tumour development. Conventional cytogenetics has identified several nonrandom copy number alterations (CNA) in uveal melanomas (UVM), which include monosomy 3, chromosome 6 abnormalities and gain of 8q. To gain further insight into the CNAs and define the regions involved more precisely we analysed 18 primary UVMs using 1 Mb BAC microarray comparative genomic hybridisation (CGH). Our analysis showed that the most common genomic imbalances were 8q gain (78%), 6p gain (67%) and monosomy 3 (56%). Two distinct CGH profiles could be delineated on the basis of the chromosome 3 status. The most common genetic changes in monosomy 3 tumours, in our study, were gain of 8q11.21–q24.3, 6p25.1–p21.2, 21q21.2–q21.3 and 21q22.13–q22.3 and loss of 1p36.33–p34.3, 1p31.1–p21.2, 6q16.2–q25.3 and 8p23.3–p11.23. In contrast, disomy 3 tumours showed recurrent gains of only 6p25.3–p22.3 and 8q23.2–q24.3. Our approach allowed definition of the smallest overlapping regions of imbalance, which may be important in the development of UVM.

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Uveal melanomas (UVM) are the most common primary intraocular malignant tumours (Stang et al, 2005). Despite successful treatment of the primary tumour approximately 50% of patients develop metastatic disease, which is usually unresponsive to chemotherapy and invariably fatal (Bergman et al, 2003). Tumours most commonly arise in the choroids with less than 10% developing in the iris and ciliary body. UVMs are classified as spindle, epithelioid, or mixed according to their histological appearance (McLean et al, 2004).

Cytogenetic analyses and allelic imbalance studies have identified a number of recurrent chromosomal aberrations in UVM, including loss of chromosome 3, and copy number alterations (CNA) on chromosomes 6 and 8 (Tschentscher et al, 2000; Aalto et al, 2001; Naus et al, 2001).

In this study we have, for the first time sought to further refine the regions of chromosomal imbalance in UVM by analysing tumours using BAC microarray CGH. The resolution of arrays in identifying regions of chromosome imbalance is dependent upon the number and distribution of the clones. The arrays used in this study were based on a previously reported BAC clone set (Fiegler et al, 2003) and we validated the detection of CNAs in a series of sex mismatch experiments through which incorrectly annotated clones were identified and excluded from subsequent analyses. The median coverage across the genome for the 3421 BACs included on the array was one clone every 1 Mb (range 500 Kb – 4.5 Mb, excluding BACs spanning centromeres). Detection of copy number changes is influenced by factors including tumour heterogeneity and contamination with infiltrating lymphocytes. In this study, we restricted our analysis to tumours that have been verified to contain less than 10% normal cell contamination. This approach has allowed for the definition of chromosomal regions that represent the smallest overlapping regions of imbalance (SORI), which are likely to harbour oncogenes or tumour suppressor genes.

MATERIALS AND METHODS

Patient samples

The tumour samples used in this study (Table 1) were obtained from different patients attending the Liverpool Ocular Oncology Centre between 1994 and 1997. All tumours were primary lesions and the diagnosis of UVM was histologically confirmed in all cases. In the nine patients alive on the 15 August 2005, the follow-up had a median of 6.97 years, exceeding 1 and 5 years in 15 patients and 10 patients, respectively. Samples were obtained with informed consent and Local Ethical Review Board approval in accordance with the tenets of the Declaration of Helsinki.

Array CGH

DNA was extracted from tumours using the QIAamp DNA Micro Kit (Qiagen, UK) and the DNA concentration was determined using the RediPlate 96 PicoGreen dsDNA Quantitation Kit.
Both procedures were performed following manufacturers instructions. The genomic DNA arrays used in these experiments were obtained from the Cancer Research UK DNA Microarray Facility and consist of 3421 BAC and PAC clones, which provide an average genomic resolution of 1 Mb. Reference DNA (Promega, UK) and test DNA were labelled separately with Cy3 and Cy5 dyes (Amersham, UK) using the BioPrime Labelling Kit (Invitrogen, UK), following manufacturers instructions and as described previously (Douglas et al., 2004).

Test and reference DNAs (50 µl each) were combined and precipitated together with 100 µg of human Cot1 DNA (1 mg ml⁻¹ Invitrogen, UK) and 50 µl yeast tRNA (5 mg ml⁻¹ Invitrogen, UK). The DNA pellets were resuspended in 10 µl of sterile water prior to being mixed with 10 µl of microarray hybridisation solution (Amersham, UK) and 20 µl of deionised formamide (Sigma, UK). The reconstituted probes were then incubated at 72°C for 15 min followed by 30 min at 37°C. The probes were hybridised to BAC arrays and incubated for 48–72 h at 37°C in a humidified chamber.

### Table 1  Clinico-pathological characteristics of patients analysed

| Tumour ID | Gender | Age at diagnosis (years) | Tumour size (mm) | Cell type | Metastatic death from UVM | Chromosome 3 status |
|-----------|--------|--------------------------|------------------|-----------|---------------------------|--------------------|
| 15        | Male   | 49                       | 18.0             | Mixed     | +                         | Monosomy           |
| 18        | Male   | 72                       | 18.5             | Mixed     | +                         | Monosomy           |
| 24        | Male   | 73                       | 17.5             | Mixed     | +                         | Monosomy           |
| 33        | Male   | 50                       | 12.8             | Mixed     | –                         | Disomy             |
| 40        | Male   | 86                       | 18.8             | Mixed     | +                         | Monosomy           |
| 41        | Male   | 29                       | 11.8             | Mixed     | –                         | Disomy             |
| 43        | Male   | 67                       | 18.4             | Mixed     | +                         | Monosomy           |
| 44        | Male   | 72                       | 17.9             | Mixed     | –                         | Disomy             |
| 53        | Male   | 42                       | 20.9             | Spindle cell | –                        | Monosomy           |
| 55        | Male   | 67                       | 14.2             | Spindle cell | +                        | Disomy             |
| 59        | Male   | 57                       | 15.8             | Mixed     | –                         | Disomy             |
| 64        | Female | 42                       | 16.2             | Mixed     | –                         | Monosomy           |
| 67        | Female | 65                       | 19.7             | Mixed     | –                         | Monosomy           |
| 69        | Female | 51                       | 13.6             | Mixed     | –                         | Disomy             |
| 75        | Male   | 54                       | 22.2             | Mixed     | –                         | Disomy             |
| 76        | Male   | 57                       | 18.4             | Mixed     | +                         | Monosomy           |
| 79        | Female | 81                       | 14.2             | Spindle cell | –                        | Disomy             |
| 81        | Male   | 52                       | 12.0             | Mixed     | –                         | Disomy             |

*Mortality from bronchial carcinoma. †Mortality from noncancer related disease.

### Table 2  Overview of genetic changes

| Tumour sample | Loss | Gain |
|---------------|------|------|
| 15            | 3p26.3–q27.3, 8p23.3–p11.1 | 8q11.1–q24.3 |
| 18            | 3p26.3–q27.3, 6q11.1–q25.3, 8p23.3–p11.23 | 2p24.3–p13.2, 5p15.3–p11.1, 8q11.1–q24.3 |
| 24            | 3p26.3–q27.3, 6q11.1–q25.3, 8p23.3–p11.1 | 2p25.3–q27.3, 6p25.3–p12.1, 7p22.3–q36.3, 8q11.1–q24.3, 17p13.3–q24.3, 21q11.2–q22.3 |
| 33            | 7p22.3–p12.2, 8p23.3–p11.23 | 8q11.1–q24.3, 21q12.1–q21.3 |
| 40            | 1p36.33–p24.3, 1p31.1–q21.1, 3p26.3–q27.3, 6q16.1–q25.3 | 6p25.3–p21.2, 8q11.1–q24.3 |
| 41            | 1p51.5–p12.33 | 4p16.1–p15.1, 4q22.3–q28.1, 6p25.2–q25.3, 7p22.1–q36.3 |
| 43            | 1p36.33–p24.3, 3p26.3–q27.3, 6q12.2–q25.3, 8p23.3–p11.2, 10p15.3–q27.3, 11q1.3–q1.33, 14p13.1–p12.2, 19p13.3–q1.34, 22q11.1–q1.33 | 6p25.3–p21.2, 8q11.1–q24.3 |
| 53            | 3p26.3–q27.3, 6q16.2–q25.3, 8p23.3–p11.1 | 4p16.1–p15.1, 4q22.3–q28.1, 6p25.2–p21.2, 7p22.1–q36.3, 8q11.1–q24.3 |
| 55            | 1p36.33–q21.2, 3p26.3–q27.3, 9q21.2–q31.3, 15q1.1–q26.3 | 6p25.3–p21.2, 8q11.1–q24.3 |
| 59            | 6q11.1–q25.3 | 4p16.1–p15.1, 4q22.3–q28.1, 6p25.2–q25.3, 7p22.1–q36.3 |
| 64            | 1p36.33–p11.2, 3p26.3–q27.3, 6q13.3–q35.2, 8q21.1–q26.3 | 6p25.3–p21.2, 8q11.1–q24.3 |
| 67            | 1p36.33–q42.3, 4p1.1–q21.1, 6q25.3–q25.3, 3q21.2–q31.3, 1q11.2–q33.3, 1q11.2–q22.3, 15q1.1–q26.3, 16p13.3–q24.3, 17p11.2–q25.3, 18p11.2–q22.3, 22q11.1–q1.33 | 4p16.1–p15.1, 4q22.3–q28.1, 6p25.2–q25.3, 7p22.1–q36.3 |
| 69            | 6q16.3–q25.3, 8p23.3–p11.1 | 6p25.3–p21.2, 8q11.1–q24.3, 9p13.2–q34.3 |
| 75            | 1p36.22–p34.2, 6q35.3, 9q34.2–q34.3, 16p13.12–p12.2, 17q21.1–q21.31, 19p13.2–q13.43, 22q11.1–q1.33 | 1q21.1–q42.3, 4p16.2–p13.1, 8p23.3–q24.3, 21q11.2–q22.3 |
| 76            | 3p26.3–q27.3, 8p23.3–p11.1 | 7p22.3–q36.3, 20q13.1–q33.3, 21q22.13–q22.3 |
| 79            | 6q11.1–q25.3 | 6p25.3–p11.1 |
| 81            | 6q11.1–q25.3 | 8q11.1–q24.3, 21q11.2–q22.3 |

Highly amplified regions in bold.
Slides were washed for 15 min at 42°C in 2 × SSC, 0.1% SDS, 15 min at 42°C in 50% formamide/2 × SSC, 30 min at 42°C in 2 × SSC, 0.1% SDS and 15 min at room temperature in 0.2 × SSC, before being dried by spinning in a centrifuge for 5 min at 150 g.

Data collection and analysis

The slides were scanned using an Axon GenePix 4000A confocal scanner, each fluorescence signal was collected separately and quantified with the GenePix Pro 3.0 software (Axon Instruments, USA). Spots were defined by use of the automatic grid feature of the software and manually adjusted where necessary. The data was normalised and analysed using Normalise Suite v2.4 (Beheshi et al, 2003), regions of loss or gain were determined as those that were 2 s.d. above the mean baseline for each separate sample.

RESULTS AND DISCUSSION

The data presented here are based on the array CGH analysis of 18 UVMs. Figure 1 shows representative CGH profiles for chromosome 6, 8 and 21. Table 2 shows the chromosomal changes for all 18 tumours analysed, and Figure 2 shows the frequency of CNAs detected at the level of each chromosome arm. The overall frequency of alterations observed in the 18 tumours was higher than has been previously reported in metaphase CGH studies of UVM (Ghavzini et al, 1996; Tschentscher et al, 2000; Aalto et al, 2001; Naus et al, 2001). This reflects the greater sensitivity of array CGH.

Previous reports have stated that the most frequent anomaly in UVM is loss of an entire copy of chromosome 3 (Sisley et al, 1992; Wiltshire et al, 1993; White et al, 1998). In our analysis, however, the most common chromosomal changes identified were 8q gains (14/18; 78, 95% confidence interval: 52 – 94%), 6p gains (12/18; 78, 95% confidence interval: 41 – 87%), and monosomy 3 (10/18; 56, 95% confidence interval: 31 – 78%). Less common CNAs were loss of 1p (6/18; 33%), 6q (7/18; 39%) and 8p (8/18; 44%), and gain of 7p (5/18; 28%) and 21q (5/18; 28%).

Inspection of the CGH profiles of monosomy 3 and disomy 3 tumours delineated two tumour types; a division supported by hierarchical clustering of the CGH data (see Supplementary data) and published microarray expression data (Tschentscher et al, 2003). In all, 10 tumours had monosomy 3 and eight disomy 3. Figure 2 shows the frequency of genomic imbalance, at the chromosome arm level, stratified by chromosome 3 status. Compared to disomy 3 melanomas, monosomy 3 tumours showed more frequent loss of chromosomal material from 1p, 6q and 8p in addition to gain of 7 and 21q. Furthermore, the regions of change in disomy 3 tumours, in some cases, involved less of the affected chromosomal arms than monosomy 3 tumours (Table 2). This probably reflects increase genomic instability in monosomy 3 tumours (Myatt et al, 2000).

Previously published analyses of chromosome 3 in UVM have shown that partial deletions of monosomy 3 are detectable in approximately 50% of tumours and have defined two minimum regions of loss, 3p25.1–p25.2 and 3q24–q26 (Tschentscher et al, 2001; Parrella et al, 2003). All the tumours we analysed with loss of chromosome 3 material showed loss of the entire chromosome, thus we were not able to further refine either of these regions of deletion.

Monosomy 3 has been hypothesised to represent an early event in tumourigenesis, defining a bifurcated tumour progression pathway (Parrella et al, 1999; Hoglund et al, 2004). In our study there was an inverse relationship between monosomy 3 and gain of 6p (Fishers exact test $P = 0.06$). It has been proposed that at least two cytogenetic pathways of clonal evolution exist for UVMs, one initiated with monosomy 3 and one with gain of 6p (Hoglund et al, 2004).

UVMs characterised by monosomy 3 are associated with a greater tumour size and a poor prognosis for survival (Prescher et al, 1996; Sisley et al, 1997; Scholes et al, 2003). Although our study did not permit survivorship associated with monosomy 3 to be formally assessed, it is noteworthy that six of the 10 patients with monosomy 3 tumours developed metastatic disease compared with only one of the eight patients with disomy 3 tumours (Fishers exact test $P = 0.06$). In our study, excluding anomalies of chromosome 3, the frequency of CNAs were higher, albeit nonsignificantly, in the tumours with monosomy 3 than in
tumours with disomy 3 (Mann–Whitney test, \(P<0.10\)); 7.5 CNAs (range 2–20) and 3.5 CNAs (range 1–13) respectively. Recent work on other cancers has shown prognosis is worse when high rates of chromosome instability are a feature, probably a consequence of the accumulation of induced genetic alterations (Nakamura et al, 2003). This might explain the better prognosis observed for UVM patients with disomy 3 tumours. In our study tumours with monosomy 3 were larger than those with disomy 3 (Mann–Whitney test, \(P<0.008\); median sizes 18.4 mm (range 16.2–20.9 mm) and 13.9 mm (range 11.8–22.2 mm, respectively) and after adjusting for tumour size there was little support for a relationship between monosomy 3 status and frequency of CNAs per se (\(P=0.80\)). Although only a small number of tumours were analysed in our study this finding invites speculation that monosomy 3 may be a consequence of clonal selection during tumour progression.

To identify SORI at each region of CNA, genomic distances spanning regions of imbalance were determined separately for monosomy 3 and disomy 3 tumours, at the resolution of individual BAC clones. The data from tumours in each group were then compared and the SORI defined for each chromosome. The SORI involving at least two of the 10 monosomy 3 tumours or two of the eight disomy 3 tumours are shown in Table 3. The SORI data described here (Table 3) corroborate the previously identified regions of imbalance reported to be associated with UVMs (Aalto et al, 2001; Naus et al, 2001), specifically, 6p (6p25.1–p21.2), 6q (6q16.2–q25.3) and 8p (8p23.3–p11.23). Furthermore, our SORI data delineates a number of additional minimal regions of imbalance in monosomy 3 patients, several less than 30 Mb, as detailed in Table 3.

In addition to confirming and refining, at base pair resolution, a number of previously reported CNAs, the use of high-resolution array CGH has allowed us to accurately delineate (to within 1 Mb) a number of rarely reported chromosomal regions of abnormality in UVM. The minimum regions defined are likely to harbour genes important to the development of UVMs.

Figure 2 Summary of chromosomal losses and gains in (A) all tumours (\(n=18\)), (B) monosomy 3 (\(n=10\)) and (C) disomy 3 (\(n=8\)) tumours. Proportion of tumours of each type with gain and loss at the level of the chromosome arm are shown by black and grey bars, respectively.

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Table 3 Smallest overlapping regions of imbalance found by array CGH for patients displaying (a) monosomy 3 (n = 10) and (b) disomy 3 (n = 8), in addition, to the genes either over expressed or under expressed with in these regions

| Affected region | Smallest overlapping region (SOFI) | SORI incidence | Chromosome position in base pairs Mb size | Approximate number of genes | Genes reported to be differentially expressed in UVM* |
|-----------------|-----------------------------------|----------------|------------------------------------------|-----------------------------|---------------------------------------------------|
| Gains (a)       |                                   |                |                                          |                             |                                                   |
| 2p23.3 – q3.7   | 2p24.3 – p3.12                    | 2/10 (20%)     | 16 036 517 – 68 981 280                  | 52.9                       | 180                                               |
| 4p16.1 – p5.1   | 4p16.1 – p5.1                     | 2/10 (20%)     | 10 782 302 – 34 782 661                  | 24.0                       | 42                                               |
| 6p25.3 – p11.1  | 6p25.1 – p21.2                    | 5/10 (50%)     | 5 060 091 – 37 828 169                   | 32.7                       | 380                                              |
| 7p22.1 – q36.3  | 7p22.1 – q36.3                    | 4/10 (40%)     | 7 725 571 – 158 345 327                  | 150.6                      | 862                                              |
| 8p23.3 – q4.3   | 8p11.21 – q4.3                    | 8/10 (80%)     | 49 397 958 – 146 062 919                 | 96.7                       | 367                                              |
| 20p12.3 – q13.33| 20p12.3 – q13.33                  | 2/10 (20%)     | 6 285 715 – 63 648 414                   | 57.3                       | 414                                              |
| 21q1.12 – 22q.23| 21q1.21 – q21.3                   | 4/10 (40%)     | 24 509 084 – 28 625 616                  | 4.1                        | 11                                               |
| 21q22.13 – q22.3| 21q22.13 – q22.3                  | 4/10 (40%)     | 34 482 466 – 46 796 424                  | 12.3                       | 135                                              |
| 3p26.3 – q27.3  | 3p26.3 – q27.3                    | 10/10 (100%)   | 186 817 – 198 819 498                   | 198.6                      | 944                                              |
| Gains (b)       |                                   |                |                                          |                             |                                                   |
| 6q11.1 – q25.3  | 6q11.1 – q25.3                    | 6/10 (60%)     | 96 260 894 – 170 637 357                 | 74.3                       | 29                                               |
| 8p23.3 – p11.1  | 8p23.3 – p11.23                   | 3/10 (30%)     | 47 774 434 – 157 173 110                 | 34.8                       | 160                                              |
| 15q11.2 – q26.3 | 15q11.2 – q26.3                   | 3/10 (30%)     | 20 403 149 – 97 832 749                  | 79.4                       | 354                                              |
| 16p13.12 – 16p12.2| 16p13.12 – 16p12.2               | 2/10 (20%)     | 13 992 248 – 21 963 275                  | 8.0                        | 57                                               |
| 22q1.11 – 22q3.31| 22q1.11 – 22q3.31                | 2/10 (20%)     | 15 615 802 – 44 352 475                  | 28.8                       | 352                                              |
| Losses (a)      |                                   |                |                                          |                             |                                                   |
| 1p36.33 – q21.3 | 1p36.33 – q21.3                   | 5/10 (50%)     | 1 059 869 – 36 259 335                   | 35.2                       | 399                                              |
| 3p26.3 – q27.3  | 3p26.3 – q27.3                    | 10/10 (100%)   | 186 817 – 198 819 498                   | 198.6                      | 944                                              |
| Losses (b)      |                                   |                |                                          |                             |                                                   |
| 6q11.1 – q25.3  | 6q11.1 – q25.3                    | 7/10 (87.5%)   | 90 997 – 19 131 250                     | 18.2                       | 81                                               |
| 8p23.3 – p11.1  | 8p23.3 – p11.2                     | 3/10 (30%)     | 108 867 491 – 146 062 919               | 37.2                       | 164                                              |
| 9p13.2 – 9q21.33| 9p13.2 – 9q21.33                   | 2/25 (25%)     | 28 840 514 – 79 219 038                  | 50.4                       | 127                                              |
| Gains Unregulated|                                 |                |                                          |                             |                                                   |
| 6q11.1 – q25.3  | 6q11.1 – q25.3                    | 7/8 (87.5%)    | 90 997 – 19 131 250                     | 18.2                       | 81                                               |
| 8q23.2 – q24.3  | 8q23.2 – q24.3                    | 6/8 (75%)      | 108 867 491 – 146 062 919               | 37.2                       | 164                                              |
| 9p13.2 – 9q21.33| 9p13.2 – 9q21.33                   | 2/5 (25%)      | 28 840 514 – 79 219 038                  | 50.4                       | 127                                              |
| Losses Downregulated|                                |                |                                          |                             |                                                   |
| 6q14.1 – 6q25.3 | 6q14.1 – 6q25.3                   | 3/8 (37.5%)    | 97 763 086 – 170 637 357                | 72.9                       | 291                                              |
| 8q23.3 – p11.23 | 8q23.3 – p11.23                   | 2/8 (25%)      | 47 774 644 – 35 173 110                  | 34.8                       | 160                                              |

*Data extracted from Dunne et al, 1998; Hendrix et al, 1998; Hurks et al, 2000; Onken et al, 2004; Radosevich et al, 2004; Tsentscher et al, 2003; van der Velden et al, 2003; Van Ginkel et al, 1998; Zuidervaart et al, 2003.
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