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Chromosomal Gains and Losses in Uveal Melanomas Detected by Comparative Genomic Hybridization[1]

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ABSTRACT

Eleven uveal melanomas were analyzed using comparative genomic hybridization (CGH). The most abundant genetic changes were loss of chromosome 3, overrepresentation of 6p, loss of 6q, and multiplication of 8q. The smallest overrepresented regions on 6p and 8q were 6pter→p21 and 8q24→qter, respectively. Several additional gains and losses of chromosome segments were repeatedly observed, the most frequent one being loss of 9p (three cases). Monosomy 3 appeared to be a marker for ciliary body involvement.

CGH data were compared with the results of chromosome banding. Some alterations, *e.g.*, gains of 6p and losses of 6q, were observed with higher frequencies after CGH, while others, *e.g.*, 9p deletions, were detected only by CGH. The data suggest some similarities of cytogenetic alterations between cutaneous and uveal melanoma. In particular, the 9p deletions are of interest due to recent reports about the location of a putative tumor-suppressor gene for cutaneous malignant melanoma in this region.

INTRODUCTION

Uveal melanoma (ciliary body and choroid) is the most common primary intraocular tumor in adults with an incidence of six to seven cases per one million people per year in North America (1). The etiology is unknown. Chemical agents (2), viruses (3), UV radiation, trauma, and nevi have been implicated in its development (1). Although uveal melanoma is not considered to be an inherited disorder, there are 14 families documented in the world literature with at least two members having this disease (1, 4).

Recently, several cytogenetic analyses of uveal melanomas have been reported, demonstrating the occurrence of monosomy 3, i(8)(q10), trisomy 8, multiplication of 6p, and a loss of the long arm of chromosome 6 in a nonrandom fashion (5–13). Between uveal melanoma involving the ciliary body and choroidal melanomas, differences in the frequencies of aberrations were observed for chromosomes 3 and 8 and accounted for the different clinical behavior of tumors at these sites (10). Molecular genetic studies revealed loss of alleles on chromosome 3 and multiplication of chromosome 8 alleles (14). Immunohistochemistry indicated high level expression of mutant p53 (15) and c-Myc protein (16).

In this study, we investigated 11 uveal melanomas with the recently introduced technique of CGH[5] (17). With CGH, differentially labeled tumor and normal DNA are hybridized simultaneously to normal metaphase chromosomes. Regions of gains or losses within the tumor DNA can be identified by an increased or decreased color ratio of the two fluorochromes used for the detection of hybridized DNA sequences along these reference chromosomes (17–23).

The comparison of the results of CGH and banding analysis revealed some unexpected findings. When compared with chromosome aberrations reported for cutaneous melanomas (24–35), our results indicate some similarities between cutaneous and uveal melanoma, hinting at the involvement of several identical genes.

MATERIALS AND METHODS

Clinical and Pathological Data. Clinical and histological data of patients with uveal melanoma are summarized in Table 1.

DNA Probes and Labeling Procedures. Total genomic DNA probes were labeled with digoxigenin-11-dUTP or biotin-11-dUTP using standard nick-translation procedures (36). The tumor DNA was obtained from fresh-frozen material.

CGH. CGH was done as described previously (18, 20) with minor modifications. Briefly, 100–200 ng of biotinylated tumor DNA was mixed with the same amount of normal male digoxigenin-labeled reference DNA and hybridized to reference metaphase spreads (46,XY) in the presence of 50 μg *Cot*1-DNA and 50 μg sonicated salmon DNA. Hybridization was allowed for 4 to 5 days. Probe detection was carried out as described (18, 20).

Digital Image Analysis. Image acquisition and image processing were performed as detailed in (18, 20). Briefly, an epifluorescence microscope (Zeiss Axiophot) equipped with a cooled, charged coupled device-camera (Photometrics, Tucson, AZ; Kodak 1400 chip) was used. Using the appropriate filter sets, gray level images were taken separately for each fluorochrome. Chromosomes were identified using the fluorescence banding pattern obtained after DAPI staining. FITC and TRITC fluorescence were specific for the tumor and the control genome, respectively. Fluorescence FITC:TRITC pixel-by-pixel ratio images (Fig. 2, *A* and *B*) were calculated as described (18, 20). Briefly, a symmetrical look-up table was used for visualization of the pixel-by-pixel FITC:TRITC ratios. The thresholds could be chosen arbitrarily since they were used for the visualization of over- and underrepresented DNA segments only.

The determination of over- and underrepresented DNA segments was done by FITC:TRITC average ratio profiles (Fig. 3). These average FITC:TRITC ratio images were calculated from at least 10 metaphases and have fixed thresholds which were tested by control experiments using normal DNA and DNA from cell lines with known numerical aberrations. The *central line* in the profiles represents the modal fluorescence ratio value measured for all reference metaphase spreads. The *left* and the *right lines* correspond to the theoretical ratio value for a monosomy or trisomy, respectively, in 50% of the cell population. These thresholds were tested for sensitivity and specifity in a great number of different hybridizations (more than 100) made by several experimenters. The procedure consists of calculation of the medial axis of each chromosome within the DAPI image, calculation of FITC and TRITC profiles along individual chromosomes, and as a last step, an averaging of individual chromosome ratio profiles from different metaphases. The entire procedure will be described in detail elsewhere.[6]

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[5] The abbreviations used are: CGH, comparative genomic hybridization; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

[6] S. du Manoir, E. Schröck, M. Bentz, M. R. S. Joos, T. Ried, P. Lichter, and T. Cremer. Quantitative analysis of comparative genomic hybridization, submitted for publication.

Table 1 *Clinical and histological data of patients with uveal melanoma*

Patient no.	Age (yrs)	Sex[a]	Tumor basis (mm)	Tumor thickness (mm)	Cell type[b]	Ciliary body involvement	Infiltration of scleral lamellae
AM89[c,d]	28	M	18.0	11.0	Sp	Yes	Yes
AM109[c,d]	70	M	15.0	12.3	Sp	Yes	Yes
AM113[d]	45	M	15.0	8.0	Mx	Yes	No
AM115[d]	67	M	14.0	9.0	Ep	Yes	Unknown
AM145[e]	31	M	8.0	2.0	Mx	No	No
AM159	65	M	22.0	8.0	Sp	Yes	Yes
AM165	79	F	16.0	12.0	Mx	Yes	No
AM185	66	F	12.0	12.0	Ep	No	No
AM186	52	F	12.0	9.0	Mx	No	No
AM187	50	M	10.0	Unknown	Mx	No	Yes
AM189	78	F	15.0	10.0	Ep	No	Unknown

[a] F, female; M, male.
[b] Ep, epitheloid; Mx, mixed; Sp, spindle.
[c] Previously published (see Ref. 7).
[d] Previously published (see Ref. 14).
[e] Previously published (see Ref. 10).

Cytogenetic Analyses. Culturing and cell processing was performed as described (7). Culturing time depended on the proliferation activity and varied for each tumor, ranging from 1 to 8 days (Table 2).

Molecular Genetic Methods. Southern blot analysis of blood and tumor DNA, densitometric analysis, and enzymatic DNA amplification were done as described previously (14). The probe pEFD64.2 was obtained through the American Type Culture Collection. It detects a highly informative variable number of tandem repeat polymorphism at the *D3S46* locus (14).

RESULTS

CGH

CGH results of tumor AM159 are exemplarily shown in Figs. 1–3. The fluorescence DAPI banding pattern used for chromosome identification is shown in Fig. 1*A*. The FITC and TRITC fluorescence intensities allowed the generation of a pixel-by-pixel ratio image displayed as a look-up table in Fig. 2*A*. Blue represents the modal fluorescence intensity ratio between the tumor and normal reference DNA. Thus, blue represents equal copy number in the tumor and normal reference genome, because both genomes are diploid. Green indicates overrepresentation and red underrepresentation in the tumor genome. This allows the generation of a copy number karyotype, shown in Fig. 2*B*. Losses of chromosome 3, chromosome arms 6q, 8p, and 16q, as well as the distal part of the short arm of chromosome 9, 9pter→p21, are readily detectable. Chromosome arm 8q is overrepresented. An average fluorescence ratio profile calculated for each chromosome from 10 metaphase spreads is exemplified in Fig. 3. The evaluation of chromo-somal gains and losses was, in all cases, based on these ratio profiles.

A survey of all CGH results from uveal tumors of 11 patients is given in Fig. 4. Losses of genetic material are represented by *vertical lines* on the *left side* of each chromosome, whereas lines on the *right side* represent gains. Case numbers are provided on the *top* of each line to facilitate the identification of changes in individual cases.

The most frequent finding was a gain of DNA segments on chromosome 8 (7 of 11) with 8q24→qter as the smallest overrepresented segment found (AM145). The second most common finding was a gain of 6p material (6 of 11) with 6pter→p21 as the smallest overrepresented segment (AM186). Loss of chromosome 3 and loss of chromosome arm 6q were found five times each. Additional findings included loss of 9p (3 of 11; AM113, AM159, and AM165), loss of 11q23→qter (2 of 11; AM89 and AM145), loss of 16q (2 of 11; AM159 and AM165), and gain of chromosome 17 (2 of 11; AM109 and AM165). Copy number changes of several other chromosomes and chromosomal subregions were noticed once, *i.e.*, loss of 1p (AM165), gain of 1q (AM145), gain of 3q25→qter (AM187), gain of 7p21→pter (AM145), gain of chromosome 9 (AM185), gain of 11p (AM187), loss of 12p (AM186), and gains of the chromosomes 14 (AM109), 21 (AM185), and 22 (AM165).

Comparison of Banding Analysis and CGH Data

Cytogenetic banding results could be obtained for 8 of the 11 tumor samples (7, Table 2[c]), revealing multiple clonal aberrations (Table 2).

Table 2 *Culture time and abnormal chromosomal findings*

Patient no.	Culture time (days)	Aberrant karyotype (*n*)	Clonal aberrations
AM89[a,b]	8	3	47,XY,add(6)(q27),dup(8)(q21qter),+dup(8)(q21qter)
		2	47,XY,add(4)(p16),add(6)(q27),dup(8)(q21qter),+dup(8)(q21qter)
		19	47,X,−Y,add(4)(p16),add(6)(q27),dup(8)(q21qter),+dup(8)(q21qter),+mar
		12	47,X,−Y,add(4)(p16),del(6)(q13),dup(8)(q21qter),+dup(8)(q21qter),+mar
AM109[a,b]	5	6	46,XY,+der(8;21)(q10;q10),add(11)(q25),−21
		5	46,XY,+der(8;21)(q10;q10),add(11)(q25),del(11)(q23),−21
		19	46,XY,del(6)(q13q26),+der(8;21)(q10;q10),add(11)(q25),−21
AM145[c]	4	21	45,X,−Y,dic(1;6)(q44;q12),+del(6)(q22),dup(8)(q2?3qter),der(16)t(1;6;16)(16pter→16q24::1q11→1q44::6q12→6pter)
AM159[c]	1–4	3	45,XY,−3,+der(8;21)(q10;q10),add(12)(p?),−14,der(19)t(14;19)(q12;p13)
		2	46,idem,+12
AM186[c]	1–3	28	45,XX,r(6)(p25→q?),add(10)(p?),16qh+,−20,i(22)(q10)
AM187[c]	3–5	3	46,XY,dup(3)(q25qter),add(6)(q?),der(6)t(6;8)(p25;q13),add(11)(p15),add(20)(q13.3)
AM189[c]	6–7	11	73–87,XXX,<4n>,-X,−1,−1,−2,−3,−3,−9,−10,−11,−12,−15,−19,+2r,+3mar[cp11]

[a] Previously published (see Ref. 7).
[b] Previously published (see Ref. 14).
[c] G. Prescher, N. Bornfeld, W. Friedrichs, S. Seeber, and R. Becher. Cytogenetics of twelve new cases of ureal melanoma and patterns of nonrandom anomalies and isochrome formation, Cancer Genet. Cytogenet., in press.

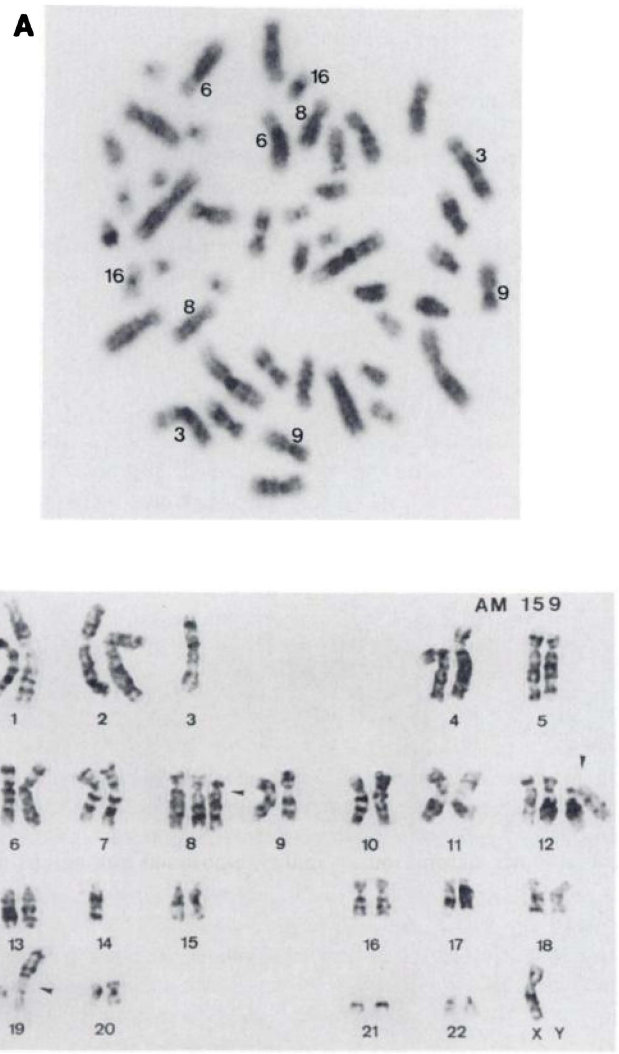

Fig. 1. CGH analysis of tumor AM159. *A*, DAPI staining of normal metaphase chromosomes used for chromosome identification in CGH experiment displayed in Fig. 2. *B*, example of a G-banding karyotype of tumor AM159. The loss of chromosome Y is not clonal.

Chromosome numbers were within the diploid range, except for case AM189 which revealed hypotetraploid chromosome counts.

For some tumors, a close correlation was observed between cytogenetic and CGH data, but marked differences were also noted. For example, compare the G-banding karyotype of tumor AM159 in Fig. 1*B* with the "copy number karyotype" in Fig. 2*B* or the average ratio profile in Fig. 3. Banding analysis and CGH revealed loss of chromosome 3. However, striking differences were noted for chromosomes 6, 8, 9, and 16. Banding analysis did not show loss of chromosome 6 material, whereas CGH demonstrated a loss of the long arm of chromosome 6. Similary, banding analysis did not suggest loss of the short arm of chromosome 8, which was revealed by CGH. All metaphase spreads evaluated by banding analysis showed two normal chromosomes 9 and 16; however, CGH showed loss of 9pter→p21 and loss of 16q. Banding analysis showed the occurrence of two additional marker chromosomes, +der(8;21)(q10;q10) and +add(12)(p?). The first marker chromosome should result in an overrepresentation of chromosome 21 material, which was not noted with CGH. The second marker chromosome should yield an overrepresentation of chromosome 12 and additional overrepresentation of DNA segments, which could not be further identified by banding analysis. However, CGH did not reveal additional chromosome 12 material. Since the long arm of chromosome 8 was the only overrepresented region in CGH analysis, one could speculate that part of the +add(12)(p?) marker chromosome could contain chromosome 8 material. Similar striking differences were found for the other tumors.

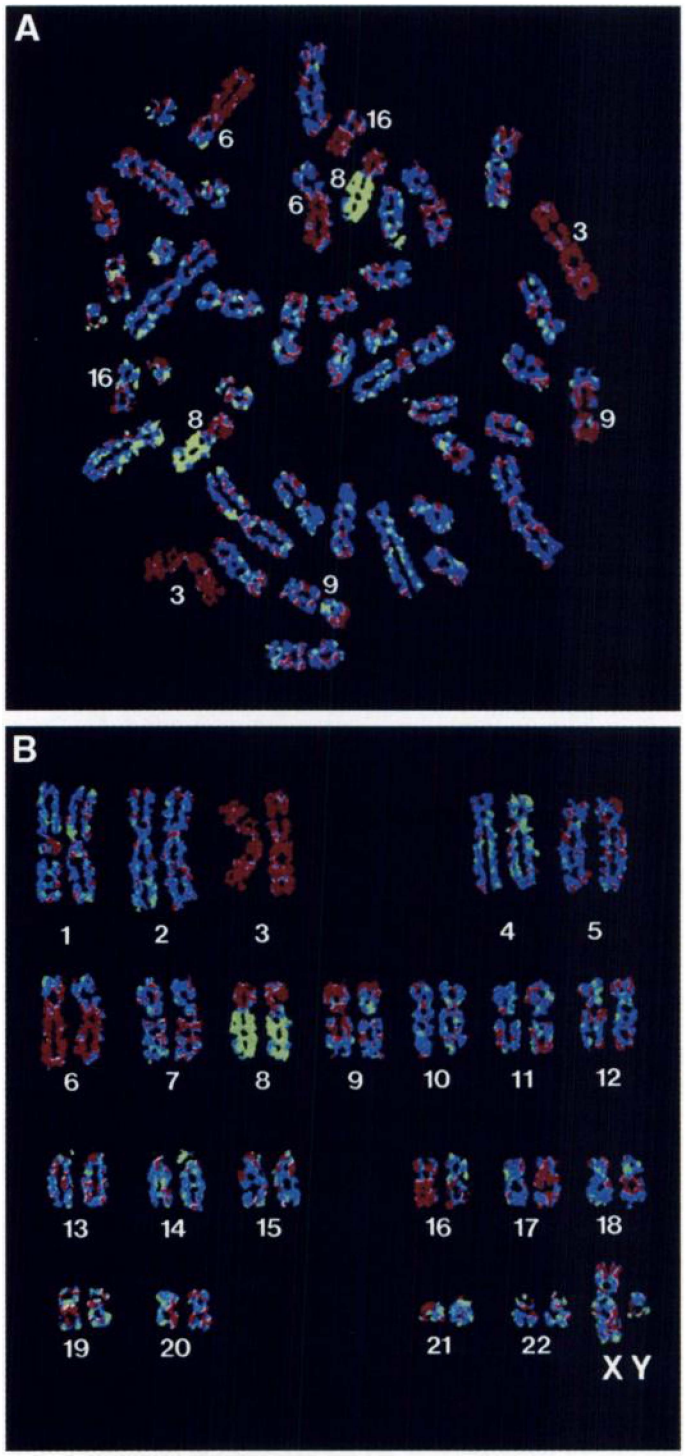

Fig. 2. *A*, fluorescence ratio image of the same metaphase spread as in Fig. 1*A* after CGH with tumor DNA AM159 and normal male reference DNA. A look-up table visualizes the pixel-by-pixel FITC:TRITC ratios. *Blue*, balanced state of chromosome material in the tumor and normal reference genome. *Green*, overrepresentation in the tumor genome. *Red*, underrepresentation in the tumor genome. The image reveals the 8q arms as overrepresented DNA segments. Other chromosomes or chromosome segments are underrepresented: chromosomes 3, 6q, 8p, 9pter→p21, 16q, and X (male patient). *B*, pixel-by-pixel ratio image of (*A*) sorted by chromosomes to facilitate the identification of numerical abnormalities.

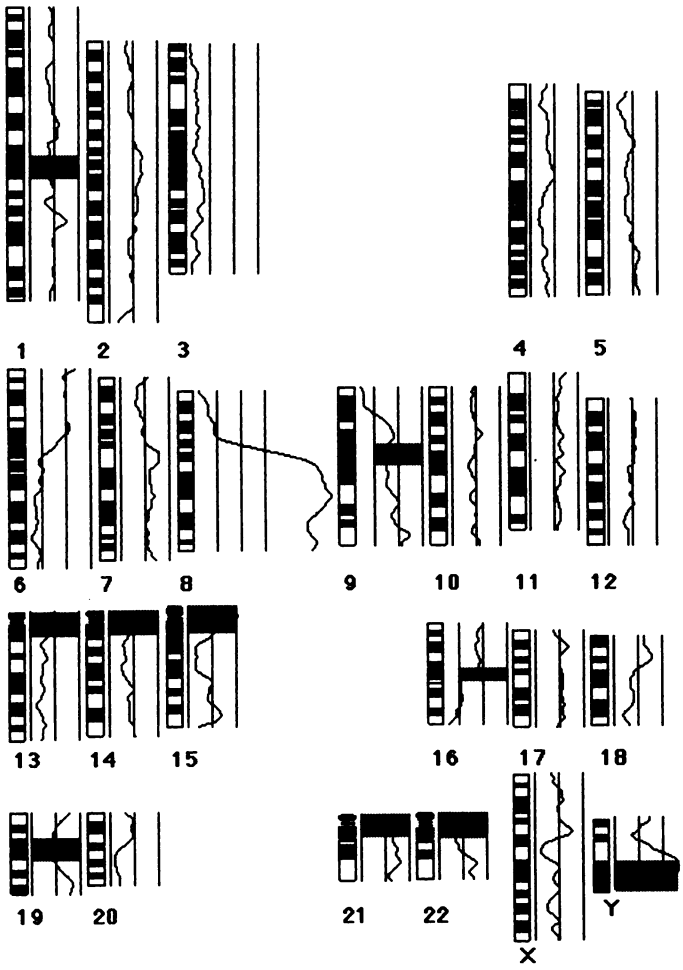

Fig. 3. Average ratio profile of tumor AM159. For details, see text and Refs. 18 and 20. Ratio profiles along the individual chromosomes are shown on the *right side* of each chromosome. *Left, middle,* and *right vertical lanes* represent the lower, middle, and upper threshold of the normal range. Due to the suppression with *Cot*I DNA fraction, the heterochromatic blocks (in particular the centromeric or paracentromeric regions of chromosomes 1, 9, and 16 and the p arms of all acrocentric chromosomes) yield unreliable ratio values and are excluded from evaluation. Fluorescence values defining the normal range correspond to the threshold values of Fig. 2*A* and 2*B*.

AM89. Overrepresentation of chromosome 8 was found by banding analysis and CGH. However, losses of 6q and the Y chromosome, as suggested by banding analysis, were not found with CGH. Instead, gain of 6p and loss of 11q23→qter were identified with CGH. The gain of 6p material could be attributed to a marker chromosome observed in banding analysis, the DNA content of which could not be identified unequivocally.

AM109. A gain of 8q and loss of 6q were found with banding analysis and CGH. Again, banding analysis showed a marker chromosome yielding additional DNA material, the origin of which could not be clarified. CGH revealed DNA amplifications of chromosome arm 6p and chromosomes 14 and 17. A deletion of 11q23→qter as found in banding analysis was not detectable with CGH.

AM145. Cytogenetic analysis showed overrepresentations of the long arms of chromosomes 1 and 6 and the distal part of the long arm of chromosome 8, 8q2?3→qter. All of these overrepresentations could be verified with CGH. Additionally, DNA multiplication was found on 7pter→p21. Losses indicated by banding analysis included the long arm of chromosome 6 and the Y chromosome. The loss of 6q was also noted with CGH, and additionally, loss of 11q23→qter was found. CGH did not reveal loss of the Y chromosome.

AM185. The only finding in banding analysis was a marker chromosome, add(21)(q22). CGH detected gain of chromosomes 9 and 21.

AM186. Banding analysis revealed several marker chromosomes such as r(6)(6pter→q?) and add(10)(p?). Unequivocal findings were loss of chromosome 20 and gain of the long arm of chromosome 22 due to an i(22)(q10). CGH revealed amplification of 6p and losses of 12p and the X chromosome.

AM187. Gain of 3q25→qter, 8q, and loss of 6q were observed by both methods, but CGH revealed addition gains in 6p and 11p.

AM189. Banding analysis revealed a hypotetraploid tumor with disomies or trisomies, respectively, of chromosomes 1, 2, 3, 9, 10, 11, 12, 15, and 19. Additionally, several marker chromosomes were observed. CGH found chromosomes 1, 2, 3, and 11 underrepresented but not the other chromosomes.

Molecular Genetic Results

Tumor and normal DNAs from five patients were studied using DNA polymorphisms on chromosome 3. The results of three patients (AM89, AM109, and AM113) were published previously (14). Constitutional heterozygosity was maintained in the tumor DNA from case AM145 but was lost for case AM159. Copy number changes of 8q in tumor DNA for AM89, AM109, and AM113 were also published in (14). All loss of heterozygosity studies were in full accordance with the findings of CGH.

DISCUSSION

Remarkable differences were noted between the results of banding analysis and CGH. Several reasons can be attributed to these differences. In contrast to banding analysis, CGH does not give information on a single cell basis but reveals only genetic imbalances which are present in the majority of the cells (>60%).[6] Chromosome banding analyses were carried out after *in vitro* cultivation. Cultural artifacts, *i.e.,* growth advantages resulting in a clonal shift during culture, may yield cytogenetic results which are not representative for the *in vivo* situation of the tumor. In contrast, CGH was performed with DNA directly prepared from tumor materials.

Previously, a very close concordance between CGH and chromosome banding was observed when tumor cell lines were subjected to both methods (17, 18, 20). Additionally, when comparing average fluorescence ratios with interphase cytogenetic data performed on uncultured nuclei of tumor samples from which the DNA was obtained for CGH, we found a linear correlation between the fluorescence ratios and the average signal number (21). Thus, we do not attribute the discrepancies between the results of banding analysis and CGH to inconsistencies of the CGH approach *per se*.

It is also notable that CGH detected some aberrations with a higher frequency than banding analysis. For example, gain of 6p was diagnosed in 14% (1 of 7) with banding analysis and in 46% (5 of 11) with CGH; loss of 9p was not found in any karyotype but in some 30% (3 of 11) of all cases with CGH.

The most commonly involved chromosomes detected by CGH were chromosomes 3, 6, and 8 (Fig. 4). This is consistent with a series of previous studies performed with chromosome banding (5–9, 11–13).

All tumors with loss of chromosome 3 material showed loss of the entire chromosome 3. Partial deletions of chromosome 3 which are a common event, *e.g.,* in nonpapillary renal cell carcinoma (37) or lung cancer (38), were not detected. These differences may indicate that several genes located on both arms of this chromosome may be involved in uveal melanoma, while genes involved in renal cell carcinoma and lung cancer may be restricted to the short arm.

The smallest overrepresented region on 6p was 6pter→p21 and 8q24→qter on 8q. While no candidate oncogene is known for 6p at present, the region 8q24→qter harbors the c-*myc* oncogene. Using

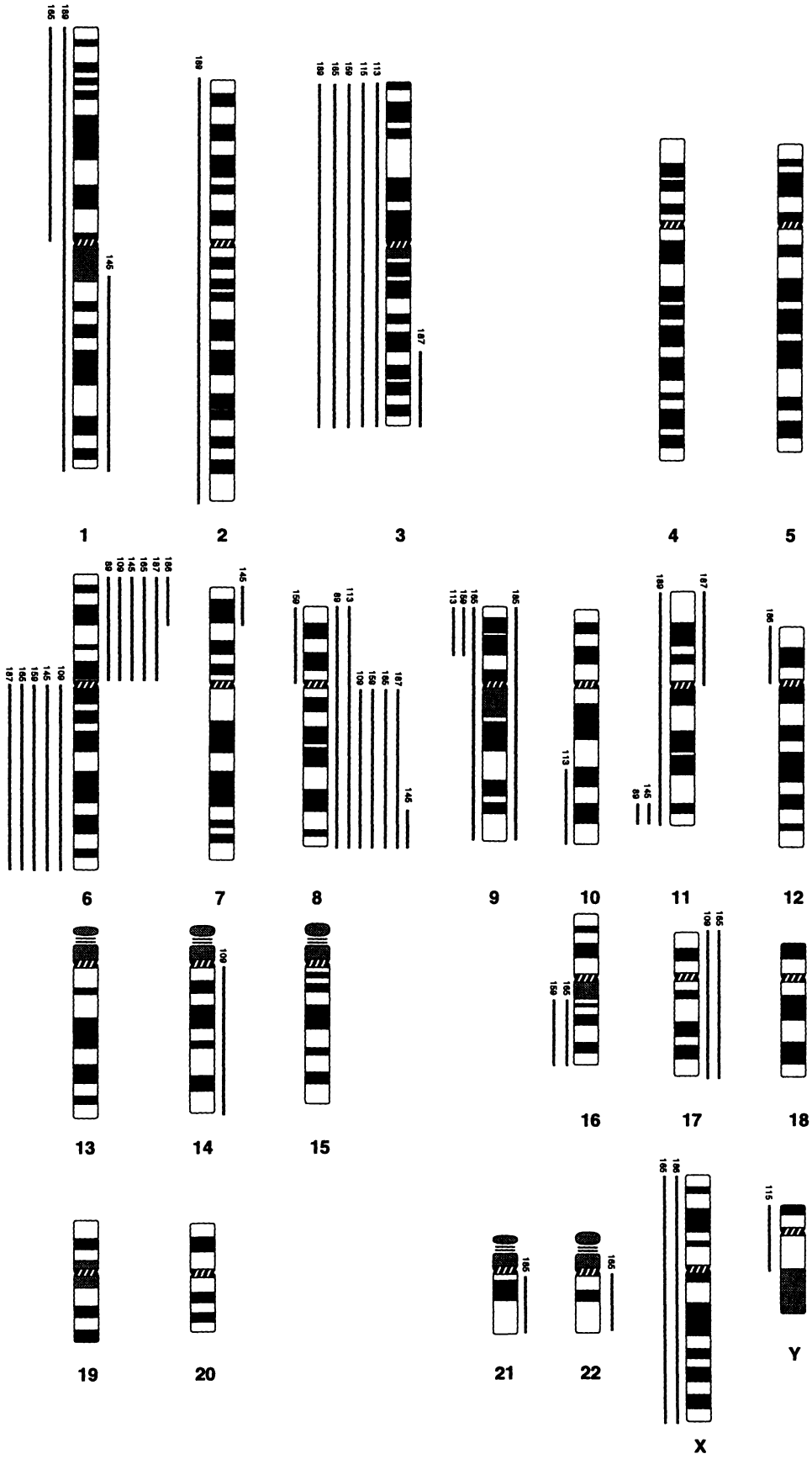

Fig. 4. Summary of all gains and losses found with CGH. *Vertical lines* on the *right side* of a chromosome represent a gain of genetic material, while *vertical lines* on the *left side* correspond to losses. Case numbers are provided on the *top* of each line.

monoclonal antibodies against the c-*myc* product, strong cytoplasmic staining has been reported for uveal melanoma, implying an involvement of this gene in cellular proliferation (16). Putative suppressor genes on 6q, a target of frequent deletion, have also not yet been identified.

No regional high-level amplifications were observed in all uveal melanomas analyzed. This correlates well with cytogenetic results where homogenously staining regions or double-minute chromosomes were never described (5–9, 11–13).

Cytogenetic differences for uveal melanomas with and without involvement of the ciliary body were reported and attributed to the different clinical behavior of these tumors (10). The interpretation of our present study has to be performed with caution due to the small number of cases, but it is interesting to note that 6p gains, 6q losses, and 8q gains occurred in both types of tumors in 50% or more of all cases. In contrast, losses of chromosome 3 (with the exception of the hypotetraploid tumor AM189), 9p, and 16q, and gains of chromosome 17 were observed in uveal melanomas involving the ciliary body only. Comparison with the literature (5–9, 11–14) indicates that chromosome 3 loss may provide a highly specific marker for ciliary body uveal melanoma and may serve to identify patients with poor prognosis (10). In our study, CGH in AM115 showed loss of chromosomes 3 and Y only. In agreement with an observation of Wiltshire *et al.* (12), who reported on a patient with ciliary body uveal melanoma showing loss of chromosome 3 as the sole visible cytogenetic aberration, it is reasonable to speculate that loss of chromosome 3 may be the first cytogenetic "hit" in the multistep pathogenesis of ciliary body uveal melanoma.

Uveal and cutaneous malignant melanomas have often been considered tumor entities with distinctly different genetic mechanisms. This was based on the fact that cytogenetic alterations of chromosomes 1, 7, 9, and 11 were frequently observed in cutaneous melanomas but rarely observed for uveal melanomas (24–34; reviewed in Ref. 35). On the other hand, overrepresentation of 6p (24, 25) and loss of 6q (26–28) were frequently noted in both entities. Recently, the locus for familial cutaneous melanoma was assigned to chromosome 9p21 by linkage analysis and physical mapping (39–42). This melanoma susceptibility gene supposedly acts as a tumor suppressor gene (42). Molecular studies showed that loss of heterozygosity on 9p was an early change in cutaneous melanoma (43). Although the cytogenetic differences between cutaneous and uveal melanoma are significant, CGH results indicate some cytogenetic similarities, suggesting the possible involvement of several identical genes. This conclusion is based on: (*a*) the fact that 6p gains and 6q losses were found with higher frequency with CGH than with chromosome banding analyses; and (*b*) the observation of 9p losses in some 30% by CGH. This is a much higher percentage than the reported alterations of 9p in the literature obtained by banding analysis (5–13) of some 8% (6 of 71 cases in Refs. 8, 11, 12, and 13). A possible explanation of this discrepancy could be provided by the explanation that tumor cells with 9p loss have a selective disadvantage during culture. AM159 provides a case in point where banding analyses revealed two entirely normal chromosomes 9, while CGH revealed a 9p loss (compare Fig. 1*B* with Figs. 2 and 3). Thus, we speculate that the number of 9p losses detectable in uveal melanomas *in vivo* may be considerably higher than in tumors cultured *in vitro* and should be considered as a nonrandom cytogenetic change in both cutaneous and uveal melanomas.

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