Genetic prognostication in uveal melanoma

Dogrusoz, M.

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Chapter 5

Radiation treatment affects chromosome testing in Uveal Melanoma

Mehmet Dogrusöz[1], Wilma G.M. Kroes[2], Sjoerd G. van Duinen[3], Carien L. Creutzberg[4], Mieke Versluis[1], Jaco C. Bleeker[1], Marina Marinkovic[1], Gregorius P.M. Luyten[1], and Martine J. Jager[1]

[1]Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands

[2]Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands

[3]Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands

[4]Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands

ABSTRACT

Purpose: The purpose of this study was to determine whether radiation treatment induces chromosomal aberrations in uveal melanoma (UM) and to evaluate which tumor features determine success of karyotyping and FISH.

Methods: Material from 327 UM-containing enucleated eyes was submitted for karyotyping, while FISH for chromosome 3 was performed in 248 samples. Thirty-six UMs had previously undergone irradiation. Karyotypes were analyzed, and the success rate of karyotyping/FISH was evaluated and compared with clinicopathologic tumor characteristics and prior irradiation.

Results: Aberrations were observed in all chromosomes, with chromosomes 1, 3, 6, 8, 13, 15, 16, and Y being altered in at least 15% of the tumors. Aberrations were more common and more complex in previously irradiated tumors (significant for chromosomes 5 [$P = 0.004$] and 13 [$P = 0.04$]). Karyotyping and FISH failed significantly more often in irradiated tumors (both $P < 0.001$). In nonirradiated cases, successful karyotyping was related to a large tumor prominence ($P = 0.004$) and a high mitotic count ($P = 0.007$). The success of FISH in these tumors was not associated with any of the studied parameters. In irradiated tumors, karyotyping succeeded more frequently in cases with a high mitotic count ($P = 0.03$), whereas FISH was more often successful in tumors with a high mitotic count ($P = 0.001$), a large diameter ($P = 0.009$) and large prominence ($P = 0.008$).

Conclusions: Karyotyping and FISH are more often successful in UMs with features characteristic of high tumor aggressiveness, whereas prior irradiation leads to multiple chromosome aberrations and to unsuccessful tests. It will be interesting to determine whether other techniques can provide reliable information on the chromosome status of previously irradiated UMs.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. It has an incidence of 5.1 per million in the United States and affects mostly Caucasians.[1,2] The median age at diagnosis is 62 years.[1] It has a strong propensity to metastasize, with up to 50% of patients eventually developing metastases, which occur predominantly in the liver.[3,4] The median survival time after detection of liver metastases is 4 to 15 months.[5]

Several clinical features and histopathologic characteristics of the primary tumor are associated with the development of metastases. These include a large tumor diameter and prominence, localization in the ciliary body, a high mitotic count, and the presence of epithelioid cells and a leukocytic infiltrate.[6–9]

Specific genetic features are also correlated with prognosis: the importance of chromosome 3 loss and chromosome 8q gain was identified first,[10–12] and subsequent studies showed that aberrations such as loss of chromosomes 1p, 6q, and 8p and gain of chromosome 6p have prognostic value as well.[13–15] Although loss of one copy of chromosome 3 is the aberration most strongly associated with poor prognosis, a combination with gain of chromosome 8q is more strongly correlated with metastatic UM than either of the two aberrations alone.[16] Besides these frequently encountered alterations, there are less often occurring ones like loss of chromosome 16.[17]

Various genetic testing methods, such as conventional karyotyping on dividing cells,[18] FISH,[19] comparative genomic hybridization (CGH),[20] single nucleotide polymorphism (SNP) testing,[21] and multiplex-ligation dependent probe amplification (MLPA),[22] can be used to determine chromosomal alterations in UM. Since 1999, genetic analysis by karyotyping and FISH has been performed in our clinic on 327 UMs obtained by enucleation. The advantage of karyotyping is that aberrations in all chromosomes are being analyzed. While some of the genetic tests analyze only specific aberrations with known prognostic value, karyotyping provides information on all chromosomes. Furthermore, karyotyping allows identification of balanced and structural chromosomal abnormalities, which do not cause a change in the quantity of genetic material. This is not possible with, for example, an SNP analysis.

We evaluated all test results to see whether we could identify other aberrations than the ones already known. We also assessed whether prior irradiation would lead to more chromosomal aberrations. We are aware of the fact that irradiation affects chromosomes in other malignancies, but we could not find much

information about the effect of irradiation on the chromosome constitution of UM and on the success rate of genetic testing.[23,24]

We investigated whether we could find any association between successful karyotyping and specific tumor characteristics and whether pre-enucleation irradiation would induce specific aberrations. We hypothesize that karyotyping and FISH succeed more often in aggressive tumors, while primary treatment by irradiation leads to an unsuccessful test.

MATERIALS AND METHODS

Patients

All patients who underwent an enucleation because of UM at the Leiden University Medical Center, The Netherlands, between 1999 and 2013, and from whom material had been submitted for chromosome testing (n = 327) were included (Fig. 1).

Enucleation was the primary treatment of 291 tumors; the remaining 36 tumors had previously been irradiated (28 received ruthenium-106 brachytherapy, 5 proton beam therapy, and 3 stereotactic radiotherapy). Of the 36 previously irradiated tumors, 18 had to be enucleated due to recurrence (after total regression), 11 due to nonresponsiveness (tumor progression after partial regression), and 7 for radiation-related complications (such as neovascular glaucoma, radiation retinopathy, radiation scleritis, and retinal detachment). Following enucleation and opening of the globe, fresh tumor material was immediately acquired and sent in for cytogenetic testing. FISH was performed on 248 samples in which karyotyping had failed or did not show a monosomy 3.The use of tumor material for research follows Dutch legal regulations, which allow the use of unused histopathologic material for research. This study adhered to the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964, ethical principles for medical research involving human subjects).

Cytogenetic Analysis

The cytogenetic evaluation of tumor samples was performed on cultured cells by a clinical cytogeneticist. Cells from the tumor sample were separated, washed, and placed into a flask with RPMI 1640 and 15% fetal bovine serum (Invitrogen, Breda, The Netherlands) and into another flask with Amniochrome II (Cambrix Bio Science, Verviers, Belgium). Culturing of the flasks occurred at 37°C with 5% carbon dioxide for up to 4 weeks, and, when at least 75% of the surface was

covered with cells, cells were harvested according to standard protocols. G-banding with Giemsa and trypsin metaphases was used for karyotyping. Karyograms were analyzed using the automatic karyotyping software Cytovision (Leica Biosystems, Inc., Buffalo Grove, IL, USA). The regulations of the International System for Human Cytogenetic Nomenclature (1995) were used for describing the karyotype. Karyotyping was classified as successful and reliable if at least 20 cells in metaphase were available for examination. FISH was performed with DNA probes specific for the centromere of chromosome 3 (probe: α-sat3; Cytocell, Cambridge, UK) and for region 3p24.3-p25 (probe: RP11-322M13). It was classified as successful and reliable if a minimum of 200 cells in interphase could be assessed. An example of a FISH test depicting monosomy 3 is shown in Figure 2A, while Figure 2B shows a case with disomy 3.
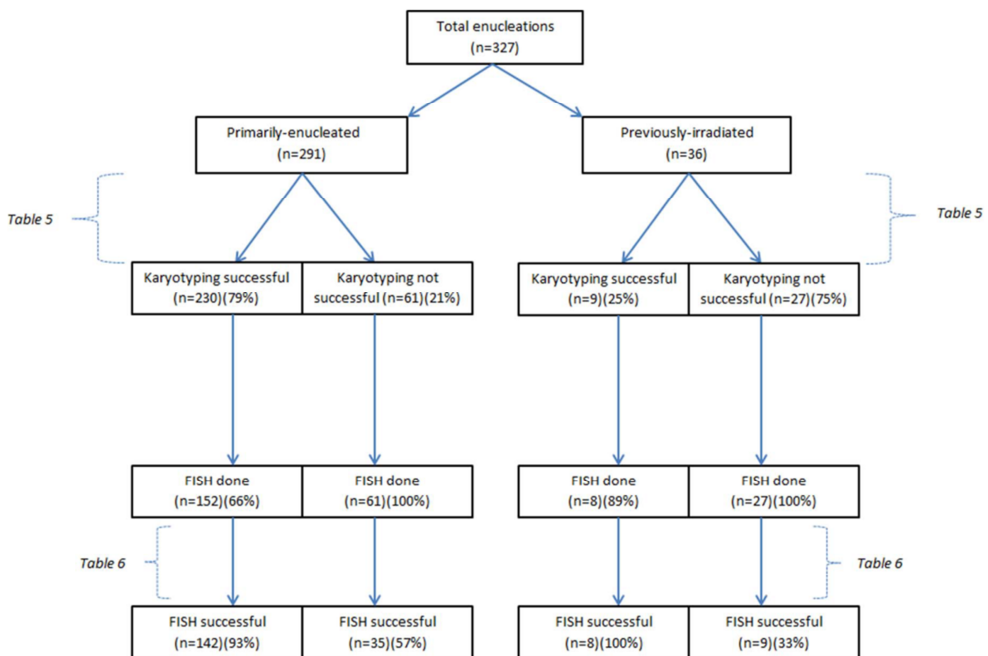
Figure 1. Flow chart depicting the numbers of tumors in which karyotyping and FISH were performed along with the success rate. Parentheses indicate the table in which the results of the respective analyses are presented.
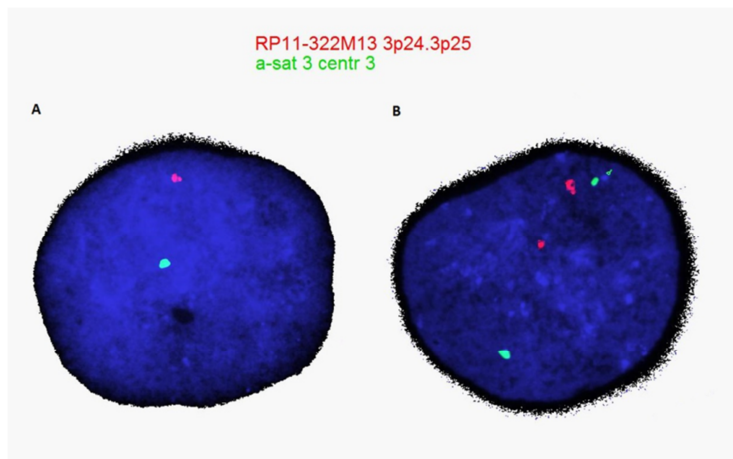
Figure 2. Examples of FISH results. (A) Monosomy 3. (B) Disomy 3. The probe for the centromere of chromosome 3 is colored green, whereas the one for the short arm (3p24.3-p25) is colored red.

Histopathologic Examination

Pathologic analysis was performed on enucleated eyes to confirm the diagnosis and determine histopathologic characteristics. Eyes were fixed in 4% neutral-buffered formalin for 48 hours and embedded in paraffin. Hematoxylin-eosin–stained sections were evaluated by a pathologist for location of the tumor in the eye, largest basal diameter (LBD, in millimeters), prominence (in millimeters), mitotic count (n/2 mm^2 at 40× magnification, eight high-power fields), and cell type (classified as mixed if at least 5% of each cell type was present and otherwise classified as spindle or epithelioid cell type). These data were registered in pathology records.

Statistical Analysis

Clinical data and the results of cytogenetic analyses and histopathologic examinations were transferred from the patient's charts and pathology reports to a database and analyzed with the SPSS statistical software package (IBM SPSS Statistics for Windows, version 20.0.0; IBM Corp., Armonk, NY, USA). Intergroup comparisons of numerical variables were performed by the Student's t-test or the Mann-Whitney U test. Associations between categorical variables were assessed by the Pearson's χ^2 test or Fisher's exact test. Differences with $P < 0.05$ were considered statistically significant, and 95% confidence intervals of the difference were calculated.

RESULTS

Frequency of Aberrations per Chromosome

Between 1999 and 2013, tumor material from 327 enucleated UMs was sent in for karyotyping. Characteristics of the cohort are shown in Table 1.

Table 1. Clinicopathologic characteristics and prior radiation treatment in relation to the success rate of karyotyping. Percentages are rounded and may not total 100. Significant *P* values are in bold.

Clinicopathologic Characteristic	Karyotyping Successful, *n* = 239	Karyotyping Not Successful, *n* = 88	P Value (95% CI of the Difference)
Gender, *n* (%)			
Male	132 (72)	52 (28)	
Female	107 (75)	36 (25)	0.53*
Age at enucleation, years			
Mean (±SD)	61.7 (±14.0)	62.5 (±13.5)	0.64† (−4.204 to 2.596)
CB involvement, *n* (%)			
No	142 (71)	58 (29)	
Yes	97 (76)	30 (24)	0.29*
LBD			
Mean (±SD)	12.2 (±3.4)	11.1 (±4.0)	**0.02† (0.191 to 1.980)**
Missing data, *n* (%)	2 (0.8)	6 (6.8)	
Prominence			
Mean (±SD)	6.9 (±2.9)	5.2 (±3.3)	**<0.001† (1.002 to 2.532)**
Missing data, *n* (%)	5 (2.1)	7 (8)	
Mitotic count			
Mean (±SD)	5.3 (±4.4)	3.6 (±3.3)	**0.002† (0.621 to 2.782)**
Missing data, *n* (%)	22 (9.2)	11 (12.5)	
Cell type, *n* (%)			
Spindle	57 (76)	18 (24)	
Mixed/Epithelioid	182 (73)	69 (28)	0.55*
Missing data, *n (%)*	0 (0)	1 (1.1)	
Prior irradiation, *n* (%)			
No	230 (79)	61 (21)	
Yes	9 (25)	27 (75)	**<0.001***

Symbols: * *Pearson's* χ^2 *test,* † *Student's t-test*

Karyotyping was successful in 239 cases (73%). We analyzed these karyotypes and observed aberrations in all chromosomes, with aberrations in chromosomes Y, 1, 3, 6, 8, 13, 15, and 16 being present in at least 15% of cases. For all chromosomes, except for chromosomes 20 and Y, irradiated tumors had more aberrations than nonirradiated tumors (Fig. 3). The difference in aberration frequency reached significance for chromosomes 5 and 13 (*P* = 0.004 and *P* = 0.04, respectively). Aberrations of chromosome 3 were present in 47% of the nonirradiated tumors

and 67% of irradiated ones (P = 0.32).
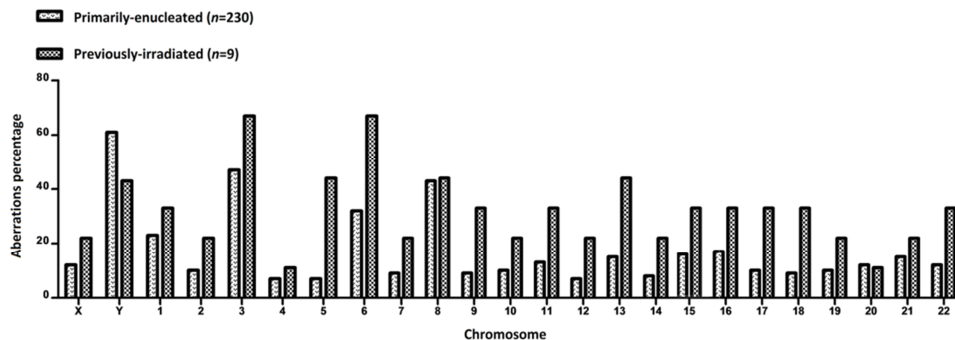
Figure 3. Aberrations percentage per chromosome, comparing primarily enucleated and previously irradiated tumors. A significantly increased frequency of aberrations was noticed for chromosomes 5 and 13 in the previously irradiated group.

Karyotypes of Irradiated Tumors

Chromosomal aberrations were not only more frequent in irradiated tumors but were also quite extensive and complex. Aneuploidy was observed in eight of the nine irradiated tumors in which karyotyping succeeded. These karyotypes are shown in Table 2, along with clinical characteristics of the tumors, the time interval between irradiation and enucleation, and the reason for enucleation (Table 3). The karyotype of tumor 07-037 is shown as an illustration in Figure 4.

Success Rate of Karyotyping in Relation to Tumor Characteristics and Prior Radiotherapy

As karyotyping was only successful in 73% of cases, we determined whether certain clinicopathologic tumor characteristics and prior irradiation were associated with success (Table 1; Fig. 1). Tumors with successful karyotyping were larger in basal diameter and prominence, with a mean of 12.2 and 6.9 mm, respectively, compared with 11.1 and 5.2 mm in the group with unsuccessful karyotyping (P = 0.02 for LBD, P < 0.001 for prominence), and had a higher mitotic count (a mean of 5.3 compared with 3.6; P = 0.002).

Prior radiation treatment had a major influence, as karyotyping succeeded in 79% of nonirradiated tumors and in only 25% of irradiated ones (P < 0.001). Irradiated tumors were found to be significantly smaller in diameter and prominence than nonirradiated cases (P = 0.02 and P < 0.001, respectively), and their mitotic count was also lower (P = 0.001; Table 4).

Table 2. Karyotypes of the nine previously irradiated tumors in which karyotyping succeeded.

Tumor ID	Karyotype
06-037	48~81,X,-X,add(3)(p25),add(5)(p15),-6,+7,+8,add(8)(q24),+9,add(9)(p23), add(9)(q34),-10,-13,-14,add(15)(p12),-16,-17,-17,-19,+20,-21,- 22,+7~16mar,inc[cp7]/46,XX[12]
07-039	Very complex aberrant karyotype. It was not feasible to provide a complete karyotype due to the bad quality of the chromosomes.
07-010	46-48,add(1)(p34),add(2)(p14),add(3)(q11),add(4)(q28),+5,add(8)(q22),add(8)(p11)[cp12]/ 46,X,-Y,+X,add(1)(p34),add(6)(q25),dup(11)(q14q22)[cp7]/ 46,XY,t(1;11)(p34;q25)[3]/ 46,X,t(Y;9)(p22;?q11)[2]/ 46,XY,add(1)(p32),?add(1)(p32),add(2)(p14),del(3)(q22),del(4)(q28)[2]/ 46,XY[3]
07-015	49-50,XX,add(1)(p12),+add(1)(p12),add(3)(q12),i(8)(q10),+i(8)(q10),add(15)(q1?5),add(16)(q21),+17,+mar[2]/ 82-95,XXXX,-X,add(1)(p12)x3,add(1)(q21)x3,-2,add(2)(q2?2),add(3)(q12)x2,-6,-10,-11,-12,+13,-15,-15,-16,+17,-18,-19,-19, 21,+7-13mar[cp6]/ 46,XX[6]
11-037	46,XY,inc[3]
10-010	45,X,-Y,i(6)(p10)[7]/ 47,X,-Y,i(6)(p10),+i(6)(p10),+22[2]
07-013	44,XY,del(2)(q3?5),der(3)add(3)(p25)add(3)(q2?7),add(3)(q12),-5,add(6)t(q2?6),del(7)(p11), der(9;15)(q10;q10),add(12)(p11),-13,-14,-15,-18,+4mar[2]/ 46,X,-Y,+mar[27]
10-005	45,X,-Y,add(1)(p3?6),?inv(3)(p1?2q2?1),add(5)(q3?3),add(13)(q2?1),add(17)(q2?1),add(18)(q2?1),add(18)(q2?1)[10]
07-037	82~95,XXYY,-3,i(6)(p10)x2,+8,i(8)(q10)x4,+11,-16,-16, −22,+1~8xmar[cp5]/ 88-89,XXYY,i(6)(p10)x2,+8,i(8)(q10)x4,-22[cp2]/ 46,XY[2]

Necrosis was more frequently observed in irradiated tumors (33% opposed to 22%). However, this difference was not significant. There was no variation in the frequency of necrosis between irradiated tumors with and without successful karyotyping (data not shown). All irradiated UMs contained melanophages.

As differences between irradiated and nonirradiated tumors may influence other comparisons, we analyzed both groups separately and explored which factors determined the success rate of karyotyping within each of these two groups. Within the group of nonirradiated UMs, karyotyping was more often successful in thicker tumors (P = 0.004) and those with a high mitotic count (P = 0.006; Table 5). In irradiated tumors, the mitotic count (P = 0.06) was the only factor that was nearly significantly associated with successful karyotyping (Table 5).

Table 3. Characteristics of previously irradiated tumors in which karyotyping was successful. Tumors are sorted in ascending order, based on the time period from irradiation until enucleation. Tumor sizes are measured in millimeters.

Tumor ID	Tumor Diameter Before Irradiation (Ultrasound)	Tumor Prominence Before Irradiation (Ultrasound)	Tumor Diameter After Irradiation (Histology)	Tumor Prominence After Irradiation (Histology)	Time Period From Irradiation Until Enucleation, months	Type of Irradiation	Reason for Enucleation
06-037	11 × 9.8	5.0	13	4	5	Ruthenium-106	Nonresponsiveness
07-039	14.5 × 13.1	8.0	9	1,5	5	Ruthenium-106	Radiation-related complications
07-010	15.3 × 10.6	4.4	14	3	8	Ruthenium-106	Tumor recurrence
07-015	13.5 × 14.3	3.7	16	5	23	Ruthenium-106	Tumor recurrence
11-037	9.5 × 10.1	5.9	9	1	28	Ruthenium-106	Tumor recurrence
10-010	11.7 × 14.2	5.9	18	2	34	Ruthenium-106	Tumor recurrence
07-013	8.3 × 8.0	3.2	6	2	71	Ruthenium-106	Tumor recurrence
10-005	11 × 9.0	8.6	8	4	71	Ruthenium-106	Nonresponsiveness
07-037	16.0	8.0	12	7	146	Proton beam	Tumor recurrence
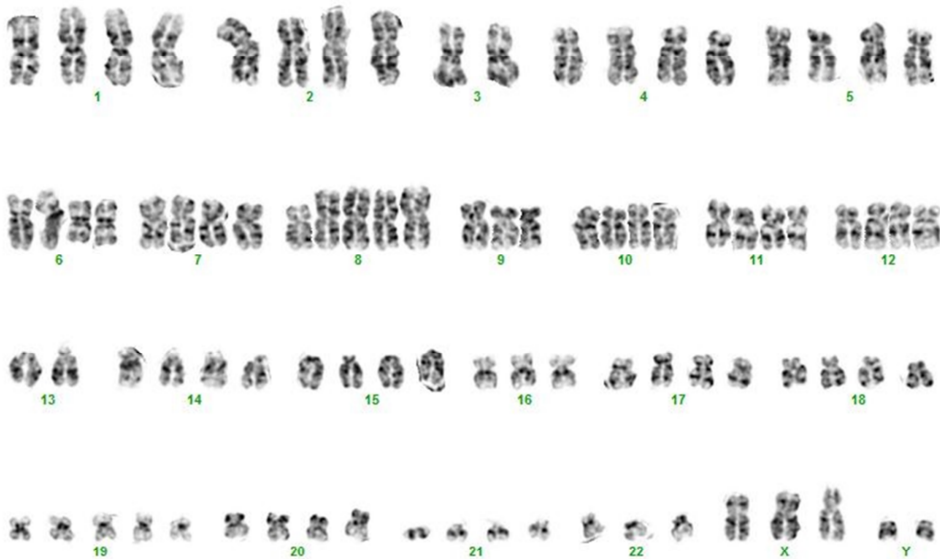
88,XXYY,+X,-3,-3,i(6)(p10)x2,+8,i(8)(q10)x4,-9,-13,-13,-16,+19,-22

07-037

Figure 4. Karyogram of the irradiated UM with tumor identification number 07-037. The karyotype of this tetraploid tumor cell is shown at the bottom.

Table 4. Comparison of histologic tumor characteristics between primarily enucleated and previously irradiated tumors. Only significant results are shown. Percentages are rounded and may not total 100.

Histologic Characteristic	Primarily Enucleated n = 291	Previously Irradiated n = 36	*P* Value
LBD			
Median (range)	12 (2–30)	10 (5–21)	0.02*
Missing data, *n* (%)	5 (2)	3 (8)	
Prominence			
Median (range)	7 (0.5–15)	3 (1–14)	<0.001*
Missing data, *n* (%)	9 (3)	3 (8)	
Mitotic count			
Median (range)	4 (0–33)	2 (0–28)	0.001*
Missing data, *n* (%)	27 (9)	6 (17)	
CB involvement, *n* (%)			
No	171 (86)	29 (15)	
Yes	120 (94)	7 (6)	0.01†

*Symbols: * Mann-Whitney U test, † Pearson's χ^2 test*

Table 5. Clinicopathologic tumor characteristics of primarily enucleated and previously irradiated UM in relation to the success rate of karyotyping. Significant *P* values are in bold.

Clinicopathologic Characteristic	Primarily Enucleated, *n* = 291			Previously Irradiated, *n* = 36		
	Karyotyping Successful *n* = 230	Karyotyping Not Successful *n* = 61	*P* Value	Karyotyping Successful *n* = 9	Karyotyping Not Successful *n* = 27	*P* Value
Gender, *n* (%)						
Male	125 (79)	33 (21)		7 (27)	19 (73)	
Female	105 (79)	28 (21)	0.97*	2 (20)	8 (80)	0.67*
Age at enucleation, years						
Median (range)	62.5 (12.8–89.6)	64.4 (8.7–85.4)	0.91†	63.8 (38.6–73.5)	66.1 (41.6–83.7)	0.37†
CB involvement, *n* (%)						
No	134 (78)	37 (22)		1 (14)	6 (86)	
Yes	96 (80)	24 (20)	0.74*	8 (28)	21 (72)	0.65‡
LBD						
Median (range)	12 (2–30)	11 (4–20)	0.17†	12 (6–18)	10 (5–21)	0.27†
Missing data, *n* (%)	2 (1)	3 (5)		0 (0)	3 (11)	
Prominence						
Median (range)	7 (0.5–15)	5 (1–12)	**0.004†**	3 (1–7)	3 (1–14)	1†
Missing data, *n* (%)	5 (2)	4 (7)		0 (0)	3 (11)	
Mitotic count						
Median (range)	4 (1–33)	3 (0–14)	**0.006†**	4 (2–28)	2 (0–15)	0.06†
Missing data, *n* (%)	19 (8)	8 (13)		3 (33)	3 (11)	
Cell type, *n* (%)						
Spindle	54 (79)	14 (21)	0.93*	3 (43)	4 (57)	0.34‡
Mixed/ Epithelioid	176 (79)	47 (21)		6 (21)	22 (79)	

*Symbols: * Pearson's χ² test, † Mann-Whitney U test, ‡ Fisher's exact test*

Success Rate of FISH in Relation to Tumor Characteristics and Prior Radiotherapy
Prior radiation treatment was found to be a limiting factor for FISH as well. Of the 35 irradiated tumors in which FISH was attempted, only 49% succeeded compared with 83% of the 213 nonirradiated cases (*P* < 0.001; data not shown). We analyzed the association of tumor features with the success rate of FISH separately for nonirradiated and irradiated tumors.

None of the studied tumor parameters was associated with successful FISH in nonirradiated cases. On the contrary, a large LBD, large prominence, and high

mitotic count were related to successful FISH in irradiated tumors (P = 0.009, 0.008, and 0.001, respectively; Table 6).

Table 6. Clinicopathologic tumor characteristics of primarily enucleated and previously irradiated tumors in relation to the success rate of FISH. Significant P values are in bold.

Clinicopathologic Characteristic	Primarily Enucleated Tumors in Which FISH Was Done, n = 213			Previously Irradiated Tumors in Which FISH Was Done, n = 35		
	FISH Successful n = 177	FISH Not Successful n = 36	P Value	FISH Successful n = 17	FISH Not Successful n = 18	P Value
Gender, n (%)						
Male	100 (83)	21 (17)		11 (44)	14 (56)	
Female	77 (84)	15 (16)	0.84*	6 (60)	4 (40)	0.47‡
Age at enucleation,years						
Median (range)	62.1 (12.8–89.6)	63.9 (8.7–84.8)	0.95†	65.5 (38.6–83.7)	62.7 (41.6–79.8)	0.69†
CB involvement, n (%)						
No	118 (86)	19 (14)		14 (48)	15 (52)	
Yes	59 (78)	17 (22)	0.11*	3 (50)	3 (50)	1‡
LBD						
Median (range)	12 (5–30)	12 (4–18)	0.74†	12 (6–21)	8 (5–13)	**0.009†**
Missing data, n (%)	4 (2.3)	1 (4.8)		0 (0)	3 (16.7)	
Prominence						
Median (range)	7 (0.5–15)	5 (1.5–10)	0.39†	4 (1–14)	2 (1–8)	**0.008†**
Missing data, n (%)	6 (3.4)	2 (9.5)		0 (0)	3 (16.7)	
Mitotic count						
Median (range)	4 (0–20)	4 (0–14)	0.57†	4.5 (0–28)	1 (0–4)	**0.001†**
Missing data, n (%)	17 (9.6)	4 (19)		3 (17.6)	2 (11.1)	
Cell type, n (%)						
Spindle	48 (80)	12 (20)		3 (43)	4 (57)	
Mixed/ Epithelioid	129 (84)	24 (16)	0.45*	14 (52)	13 (48)	1‡

*Symbols: * Pearson's χ^2 test, † Mann-Whitney U test, ‡ Fisher's exact test*

Success Rate of Karyotyping and FISH in Relation to the Reason of Enucleation of Irradiated Tumors

As there are various reasons for enucleating an eye following prior irradiation, we

analyzed whether there was a relation between the reason for enucleation and the success of karyotyping and FISH.

Among irradiated tumors enucleated for tumor recurrence (n = 18), karyotyping was successful in six cases (33%). This rate was 18% (2/11) for nonresponsive tumors and 14% (1/7) for tumors enucleated due to radiation-related complications. FISH succeeded in 56% (10/18) of the recurrent tumors, 36% (4/11) of nonresponsive cases, and 43% (3/7) of tumors enucleated because of radiation-related complications. Differences in success rate between the various causes of enucleation were not significant for karyotyping or FISH.

DISCUSSION

In our clinic, karyotyping and FISH have been applied to improve the chances of a successful genetic typing of UMs. FISH has also been utilized by many other centers as a prognostic test and remains an excellent alternative to other more expensive tests.[25] A disadvantage of FISH is, however, that only alterations affecting the targeted chromosomal region can be detected.[26] Karyotyping on the other side provides information on aberrations in all chromosomes and furthermore allows identification of balanced and structural chromosomal abnormalities. However, only alterations larger than 3 to 5 Mb in size can be reliably detected.[27]

We observed aberrations in a wide variety of chromosomes, especially the ones reported previously (chromosomes 1, 3, 6, and 8). Aberrations were more frequent and more complex in irradiated cases. A significant increase in aberration frequency for chromosomes 5 and 13 was found.

The type of aberrations that we observed in nonirradiated cases were similar to those described previously in two reports on 120 and 152 karyotyped cases.[15,17] Most studies reporting on other techniques for chromosomal analysis provide only information on nonirradiated cases, in which a successful analysis of chromosomes 3, 6, and 8 is often possible. However, we were specifically interested in the influence of irradiation on chromosomal analysis.

When looking at the nonirradiated tumors, a large tumor prominence as well as a high mitotic count was related to a successful test. The association with a high mitotic count was especially expected, as success of conventional karyotyping depends on the presence of metaphasic cells. The association between larger tumor size and successful karyotyping is less unequivocally explainable since a larger tumor size does not necessarily imply a higher mitotic count. Although

tumor diameter and prominence were significantly correlated to mitotic count in our cohort, this correlation was not strong (data not shown).

Pre-enucleation radiation treatment was, as we hypothesized, strongly associated with unsuccessful karyotyping. We expected that tumor shrinkage and necrosis caused by irradiation, as shown in previous studies by Saornil et al.,[28,29] would leave an insufficient number of dividing cells available for karyotyping. Indeed, cell proliferation has been shown to be lower in irradiated tumors: posterior uveal melanomas treated by Ru-106 brachytherapy were found to have a lower expression of the PC-10 cell proliferation marker in comparison to primarily enucleated melanomas.[30] Ki-67 scores and mitotic activity were also found to be significantly lower in irradiated tumors.[31,32] We also noticed that the mitotic count is lower in irradiated tumors and that high numbers are associated with successful karyotyping in nonirradiated as well as in irradiated tumors. Alternatively, irradiated tumors may already have a lower mitotic count because smaller tumors are selected for radiation treatment. As a matter of fact, a small tumor diameter was associated with a lower number of mitoses in our cohort (data not shown). The radiobiologic effects of irradiation at the cellular level could also play a role in the failure of karyotyping. Tumor cells still remaining after irradiation may have accumulated complex radiation-related chromosomal aberrations to such an extent that it probably has rendered them incapable of dividing and induced cell cycle arrest and senescence. A frequently occurring complex aberration of the chromosomes in tumor cells is aneuploidy. Aneuploidy has been found to be significantly more common in irradiated tumors.[33]

A rather special group of previously irradiated tumors are those that are enucleated because of tumor recurrence. We observed that, although not significantly different, karyotyping and FISH were more often successful in tumors enucleated because of recurrence than nonresponsiveness or radiation-related complications. Chiquet et al.[34] showed that Ki-67 scores are higher in recurrent tumors compared with those enucleated because of post–proton irradiation neovascular glaucoma. In our study population, the median mitotic count was significantly higher in irradiated tumors enucleated for tumor recurrence (data not shown). Furthermore, the new tumor arising in the case of recurrence is unaffected by irradiation and probably therefore more suitable for successful karyotyping.

As FISH testing for monosomy 3 becomes especially relevant when karyotyping does not provide a reliable result, we analyzed what determined the success of

FISH in previously irradiated cases. A large LBD, large prominence, and high mitotic count were associated with successful FISH. These are the same determinants that played a role in the success of karyotyping, but FISH provided useful information in almost 50% of cases.

We recognize no information on monosomy 3 testing was obtained in 44 of 327 (13%) of the cases. In our series, this problem occurred especially in irradiated cases. There are very few reports regarding the eligibility of cytogenetic testing in previously irradiated UMs. One study on 15 cases of irradiated choroidal melanoma used CGH and found successful results in all tumors.[35] Another study, evaluating the use of gene expression profiling after radiotherapy, showed successful results following iodine-125 plaque radiotherapy and proton beam therapy. However, this involved a case series of only three tumors.[36]

We conclude that the success of karyotyping and FISH is determined by histologic tumor features characteristic of high tumor proliferation and growth. Karyotyping revealed that aberrations can be found in all chromosomes and that the frequency increases after irradiation. An important finding is that karyotyping and FISH especially fail quite often in previously irradiated tumors, supporting the approach in which biopsies are taken prior to irradiation.[37–39] However, as more chromosome aberrations are observed after prior irradiation, one may still wish to determine the chromosome status in postirradiation enucleated eyes. It will be interesting to see how irradiation affects the outcomes of other DNA-based tests such as MLPA, SNP, or ddPCR or any RNA-based techniques such as class I/class II testing in enucleated eyes.

Competing Interests Statement
The authors declare no conflicts of interest.

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