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Genetic analysis of uveal melanoma by array comparative genomic hybridization before and after radiotherapy

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Summary

Background Genetic analysis of choroidal melanoma is frequently used to estimate the risk of metastatic spread of the tumor. Obtaining a biopsy for genetic analysis, however, can be difficult and sometimes unsuccessful. We evaluated the feasibility and accuracy of genetic testing using array comparative genomic hybridization (CGH) after radiotherapy, from tumor samples obtained by endoresection or after secondary enucleation.

Material and methods Fifteen choroidal melanoma samples obtained after radiotherapy (Ruthenium-106 plaque brachytherapy or Gamma-Knife radiosurgery) were analyzed by array CGH to detect chromosomal aberrations (monosomy 3 and trisomy 8q), and the results were compared with pre-irradiation findings in five cases.

Results Array CGH was successfully performed in all 15 cases. Time from radiotherapy to obtaining the sample for cytogenetic testing was between 14 and 879 days. Results of post-radiotherapy genetic analysis did not differ from pre-radiotherapy findings.

Conclusion Post-radiation CGH appears to be a promising option for prognostic testing if a first biopsy before radiotherapy failed or was not performed. It could be useful to avoid an additional surgical procedure before radiotherapy if vitrectomy or endoresection is planned after radiotherapy.

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Genetische Analyse von Aderhautmelanomen mittels Array-CGH vor und nach Strahlentherapie

Zusammenfassung

Hintergrund Die genetische Untersuchung von Aderhautmelanomen ist eine zunehmend häufiger eingesetzte Methode, um das Risiko der Metastasenentwicklung bei PatientInnen mit Aderhautmelanomen einzuschätzen. Die Gewinnung einer Gewebeprobe zur Durchführung der Untersuchung ist jedoch manchmal schwierig und nicht in allen Fällen erfolgreich. Wir untersuchten die Durchführbarkeit und Genauigkeit der genetischen Untersuchung von Aderhautmelanomen mittels array comparative genomic hybridization (CGH) nach Strahlentherapie, an mittels Endoresektion oder nach Enukleation gewonnenem Tumormaterial.

Material und Methodik Fünfzehn Gewebeproben von strahlentherapeutisch behandelten Aderhautmelanomen wurden mittels array-CGH auf Veränderungen an den Chromosomen 3 und 8 untersucht (Monosomie 3, Trisomie 8q). Die Ergebnisse wurden mit den in fünf Fällen vorliegenden Resultaten der genetischen Untersuchung vor Bestrahlung verglichen.

Resultate Die array CGH konnte in allen 15 Fällen nach Bestrahlung erfolgreich durchgeführt werden. Die Zeitspanne von der Bestrahlung bis zur genetischen Untersuchung lag zwischen 14 und 879 Tagen. Die Resultate der genetischen Untersuchung nach Bestrahlung unterschieden sich nicht von den Ergebnissen der in 5 Fällen vorliegenden Ergebnissen vor der Bestrahlung.

Schlussfolgerung Die array CGH von Aderhautmelanomen nach Strahlentherapie erscheint eine vielversprechende Option zur prognostischen Unteruchung in den Fällen zu sein, in denen eine Biopsie vor Bestrahlung

nicht durchgeführt wurde oder nicht erfolgreich war. Im Falle einer geplanten Endoresektion nach Bestrahlung, könnte auf einen zusätzlichen Eingiff zu Biopsie vor der Bestrahlung verzichtet werden.

Schlüsselwörter Aderhautmelanom · Genetische Untersuchung · CGH · Strahlentherapie · Endoresektion

Introduction

The eye is the most common location for non-cutaneous primary melanoma [1]. A total of 85 % of all ocular melanomas arise from the uvea, and uveal melanoma is the most common primary intraocular tumor in adults [2]. In contrast to melanoma of the skin, the frequency of uveal melanoma has not increased over the past decades [3]. Mortality is mainly due to metastasis to the liver, which occurs in up to 45 % of patients within 10 years after treatment [4]. Local treatment for intraocular melanoma, usually enucleation or radiotherapy, has a high success rate and achieves excellent tumor control, but fails to improve survival [3]. Clinical and histopathologic risk factors for the development of metastatic disease have been identified and evaluated for their prognostic significance [5]. Classical risk factors include a large tumor size, ciliary body involvement, epithelioid cell type, and extravascular matrix patterns. Over the past decade, cytogenetic studies of choroidal melanoma identified characteristic non-random chromosomal abnormalities in choroidal melanoma cells, affecting chromosomes 1, 3, 6, and 8 in up to 50 % of melanoma patients [6, 7]. Specific cytogenetic abnormalities, such as loss of one copy of chromosome 3 and amplification of the long arm of chromosome 8, have been shown to be associated with unfavorable prognosis, and are superior to traditional prognostic markers in predicting metastatic spread [8, 9]. Over the past years, cytogenetic analysis of choroidal melanoma slowly developed from a research tool into a routine clinical test [10]. Selected centers have been using genetic testing in clinical routine for more than a decade now [11]. For analysis, only a small sample of tumor tissue has to be obtained. However, the proportion of patients treated with enucleation has decreased over the past decades, and an increasing number of patients are treated with globe-preserving intent, mainly by radiotherapy [3]. Tumor tissue, therefore, has to be acquired by taking biopsies from the melanoma via a transvitreal or transscleral approach before treatment [12–14]. On the other hand, an increasing number of patients undergo vitrectomy and/or endoresection of the tumor after radiotherapy [15–17]. Cytogenetic analysis of tumor material obtained by endoresection after radiation would be a convenient method to acquire a sufficiently large tumor sample for genetic analysis. However, there is no literature available regarding the validity of commonly used genetic tests for uveal melanoma after radiotherapy. In this report, we present results of post-radiotherapy genetic testing using array comparative genomic hybridization (CGH) and compare the results with pre-radiation findings in a small series of patients with choroidal melanoma.

Materials and methods

All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 [5]. Approval of the local ethics committee was obtained to review all cases of patients with choroidal melanoma, who had pre- and/or post-radiation cytogenetic testing. All patients gave their written informed consent to evaluate the results from genetic analysis for research before the test was performed.

Biopsy technique

Since 2007, we have been offering cytogenetic testing to all our patients with ocular melanoma, following an approach similar to the one described by Damato et al. [10]. Diagnosis of choroidal melanoma was made after a comprehensive ophthalmologic examination, using indirect ophthalmoscopy and ultrasonography in all cases. Fluorescein and indocyanine green angiographies were done if necessary. All patients were sent to an oncologist or a specialist for internal medicine to rule out distant metastases. Systemic evaluation before radiotherapy included ultrasonography of the liver, magnetic resonance imaging (MRI) of the head/orbit and abdomen, and blood test, including liver parameters. To obtain tumor samples during plaque brachytherapy, we used a short 27-gauge needle connected to a 5-ml syringe via a short plastic tube (4 cm). After removal of the dummy plaque and immediately before suturing the plaque to the globe, the surgeon (Werner Wackernagel) dried the sclera with a cotton tip and then perforated the sclera in a tangential direction, to make the wound self-sealing. When entering the tumor, the needle direction was changed to almost perpendicular to the scleral surface, and suction was applied via the syringe to aspirate tumor cells. After withdrawal of the needle, a cotton tip was pressed onto the sclera to prevent bleeding. The cells were flushed from the needle tip into the syringe by aspirating balanced salt solution (BSS) and were immediately sent for genetic analysis. Before Gamma-Knife radiosurgery, specimens were obtained by 23-gauge transvitreal biopsy. The instruments were entered into the eye in typical and standardized manner [18–20]. The vitreous cutter was inserted into the melanoma, and tumor tissue was aspirated for 10–20 s. The instruments were withdrawn, and the biopsy material was aspirated from the tube into a 5-ml syringe. The trocars of the 23-gauge vitrectomy system prevented direct contact between the vitreous cutter and the sclera at the entry sites. To prevent post-operative hypotony, sclerotomies were sutured in case they did not appear to be completely self-sealing. Samples for post-radiotherapy

testing were obtained after secondary enucleation or during endoresection. After enucleation, the globe was cut along the meridian opposite the tumor base. A small sample ($2 \times 2 \times 2$ mm^3) was cut from the tumor apex, put into BSS, and sent for cytogenetic testing. Endoresection was performed by one surgeon (Andreas Wedrich) using a standard 3-port 20-gauge vitrectomy system without systemic hypotension [21]. Tumor material was aspirated into a 10-ml syringe and sent for cytogenetic testing immediately after surgery.

DNA isolation and amplification

DNA was extracted by the means of the Qiagen Mini Kit (Qiagen, Vienna, Austria) according to the manufacturer's instructions. In cases where not enough tumor material was available, cells were applied onto a polyethylene terephthalate (PET) membrane-covered microscope slide (Zeiss, Austria). Isolation of the cells of interest was carried out using a laser microdissection and pressure catapulting system (LMPC; P.A.L.M., Zeiss, Austria). The cells were selected and directly catapulted into the cap of a 200-µl Eppendorf tube containing 10 µl of digestion mix.

Whole-genome amplification of the DNA was performed using the GenomePlex Single Cell Whole Genome Amplification Kit (#WGA4; Sigma-Aldrich, Germany). After purification using the GenElute PCR Clean-up Kit (#NA1020; Sigma-Aldrich, UK), DNA concentration was determined by a Nanodrop spectrophotometer. Amplified DNA was stored at -20 °C.

Array CGH

Array CGH was carried out using a commercially available whole-genome oligonucleotide microarray platform (Human Genome CGH 44B and 60K Microarray Kit, Agilent Technologies, Santa Clara, CA). As a reference DNA, commercially available male DNA was used (Promega, Madison, WI, USA), and in case of amplified test DNA, amplified reference DNA was used. Samples were labeled with the Bioprime Array CGH Genomic Labeling System (Invitrogen, Carlsberg, CA, USA) according to the manufacturer's instructions. Briefly, 250–500 ng of test DNA and reference DNA were differentially labeled with dCTP-Cy5 or dCTP-Cy3 (GE Healthcare Corp., Piscataway, NJ, USA). Further steps were performed according to the manufacturer's protocol (version 6.0; http://www.agilent.com). Slides were scanned using Agilent's microarray scanner G2505B (Agilent Technologies), and images were analyzed using Feature Extraction and DNA Workbench software 5.0.14.

Results

In total, 15 patients had post-radiotherapy genetic analysis, either after Ruthenium-106 plaque brachytherapy (5 patients) or after Gamma-Knife radiotherapy (10 patients). In ten cases, only post-radiotherapy testing was available; 5 patients had genetic analysis done before and after radiotherapy.

Radiation dose was 100 Gy to the tumor apex for brachytherapy and 30 Gy [50 % isodose encompassing the PTV (planning target volume)] for Gamma-Knife radiosurgery in all cases.

In total, median time between radiotherapy and post-radiation cytogenetic testing for all 15 patients was 154 (range: 14–879) days . Five patients showed concurrent monosomy 3 and gains of chromosome 8, two showed monosomy 3 only, four showed only gains of chromosome 8, and four showed no changes of chromosome 3 or 8. The median observation time after radiotherapy for the eight patients without monosomy 3 was 365 (range: 40–1270) days, and as expected, none of these patients developed liver metastases. The median follow-up time for the seven patients with monosomy 3 was 733 (range: 368–1063) days. One of those seven patients developed liver metastasis 14 months after initial treatment.

Pre- and post-radiotherapy testing: before radiotherapy, two patients had transscleral fine-needle aspiration biopsy (before Ru-106 brachytherapy), and in three patients, biopsy of the melanoma was obtained by 23-gauge transvitreal biopsy (before Gamma-Knife radiosurgery). In four of those melanomas, we observed both loss of chromosome 3 and gain of the long arm of chromosome 8. One melanoma had only a gain of 8q (Table 1). Median time between radiotherapy and post-radiation genetic analysis in those five cases was 76 (range: 34–526) days.

The comparison between pre- and post-treatment results revealed unchanged status of chromosomes 3 and 8 in all cases (Table 2, Fig. 1). However, in two cases (case 1 and case 5), the breakpoints identified on chromosome 8q were slightly different before and after radiotherapy.

Post-radiotherapy testing only: 10 patients who did not have pre-operative genetic testing underwent endoresection (6 cases) or enucleation (1 case) after Gamma-Knife radiosurgery, or endoresection after brachytherapy (3 cases), and asked for cytogenetic analysis of the irradiated tumor. Array CGH analysis was successfully performed on the post-radiation material between 14 and 879 days after radiotherapy (median: 347 days) and allowed to establish the copy number status of chromosomes 3, 8, and other chromosomes. Of those 10 melanomas, 3 showed monosomy 3, 4 showed gains of chromosome 8, 1 melanoma showed both changes, and in 4 cases, chromosomal status was normal.

Conclusion

Cytogenetic testing of uveal melanoma has advanced from a research tool to a prognostic test used in daily clinical routine [10]. Until now, genetic testing has been performed and published on specimens obtained from enucleated eyes or on biopsies taken before radiotherapy

Table 1 Results of cytogenetic analysis before and after radiotherapy

| ID	Age	Initial treatment	Secondary treatment	Days after radiotherapy	Chromosome 3 status before radiotherapy	Chromosome 8q status before radiotherapy	Chromosome 3 status after radiotherapy	Chromosome 8q status after radiotherapy
01	46	GK	Endores	76	Normal	Gain	Normal	Gain
02	49	Ru-106	Endores	74	Loss	Gain	Loss	Gain
03	58	GK	Endores	526	Loss	Gain	Loss	Gain
04	65	Ru-106	Endores	34	Loss	Gain	Loss	Gain
05	49	GK	Endores	281	Loss	Gain	Loss	Gain
06	88	GK	Endores	56			Normal	Normal
07	64	Ru-106	Endores	14			Normal	Normal
08	54	Ru-106	Endores	55			Normal	Gain
09	47	GK	Enuc	154			Normal	Gain
10	66	GK	Endores	585			Loss	Normal
11	77	GK	Endores	879			Normal	Normal
12	86	GK	Endores	47			Loss	Gain
13	55	GK	Endores	322			Normal	Gain
14	48	GK	Endores	386			Normal	Normal
15	78	Ru-106	Endores	371			Loss	Normal

Initial treatment: *GK* Gamma-Knife radiotherapy, *Ru-106* Ruthenium-106 plaque brachytherapy
Secondary treatment: *Endores* vitrectomy and endoresection, *Enuc* enucleation

Table 2 Details of the results for patients with pre- and post-radiotherapy cytogenetic analysis

ID	Chromosome 3 status before/after radiotherapy	Chromosome 8q status before/after radiotherapy
01	Balanced/balanced	Gain 8q21.12-qter/gain 8q13.13-qter
02	Loss 3pter-qter/loss 3pter-qter	Gain 8q11.21-qter/gain 8q11.21-qter
03	Loss 3pter-qter/loss 3pter-qter	Gain 8pter-qter/gain 8pter-qter
04	Loss 3pter-qter/loss 3pter-qter	Gain 8p11.21-qter/gain 8p11.21-qter
05	Loss 3pter-qter/loss 3pter-qter	Gain 8p23.1-qter/gain 8p11.21-qter

only [12, 22, 23]. Our results demonstrate the feasibility of cytogenetic testing after radiotherapy and show that results of array CGH are not altered by radiotherapy.

There are several possible indications for post-radiation cytogenetic testing. First, post-radiation testing offers a chance for prognostic genetic analysis if a first attempt before radiotherapy was unsuccessful. Unsuccessful analysis has been reported in up to 25% of patients for fluorescence in situ hybridization on fine-needle aspiration biopsies [23]. A reliable salvage procedure to obtain genetic profile might become increasingly important when patients with high-risk melanoma—and only those—are to be included into adjuvant treatment trials. Second, if endoresection is planned, or the need for additional intraocular surgery is foreseeable, one
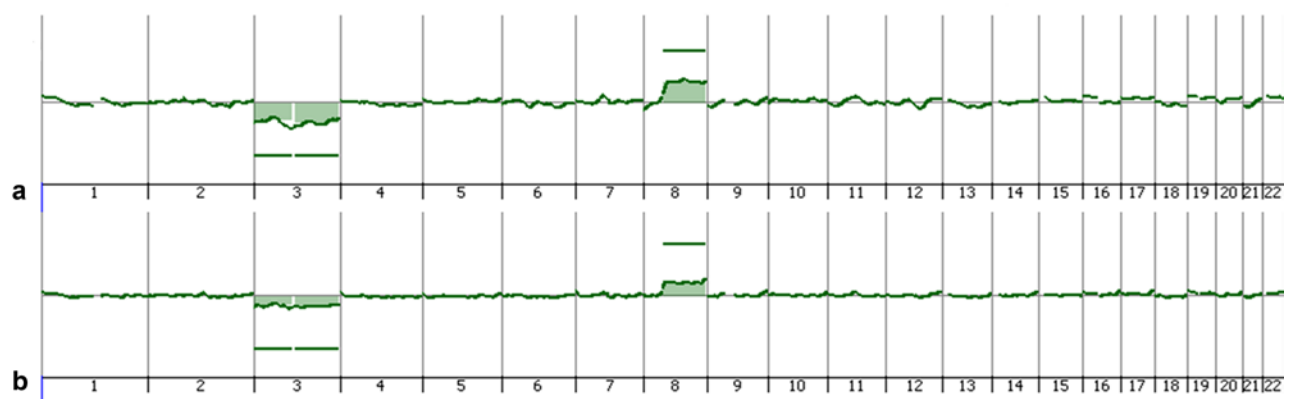
Fig. 1 Array CGH results for patient 02 (ID) before (**a**) and after (**b**) radiotherapy, showing identical results, with loss of chromosome 3 (monosomy 3) and gain of the long arm of chromosome 8. Array CGH profile is not altered by previous radiotherapy

could avoid an additional surgical procedure before radiotherapy and the possible complications resulting from tumor biopsy before radiotherapy. Endoresection has become increasingly common after radiotherapy of large uveal melanomas [15, 17]. During surgery, large tumor samples can be obtained without an additional surgical procedure and without jeopardizing visual outcome [24]. Third, post-radiation biopsy might help to avoid the hypothetical risk of spreading tumor cells into the blood stream or seeding cells in the needle tract [25]. There is no evidence supporting a hypothetical spreading of melanoma cells into systemic circulation by tumor biopsy, and a correlation between treatment modality and the amount of circulating tumor cells could not be established [26]. On the other hand, most centers still try to avoid endoresection without previous irradiation of the tumor [15, 27]. Similarly, seeding of tumor cells at the sclerotomy site seems to be a rare event. However, a few cases of extraocular extension after biopsy have been reported recently [28]. Post-radiation biopsy might help to further reduce the risk of spreading tumor cells, and thus increase the acceptance of prognostic genetic testing.

Time after radiotherapy did not appear to be a limiting factor for genetic analysis by array CGH in our case series. Even patients who had their melanoma treated several years ago, when cytogenetic analysis was not routinely performed, could perhaps be offered this prognostic test. Vital-appearing tumor cells have been found in uveal melanomas years after brachytherapy, without clinical evidence of tumor recurrence [29]. As CGH is based on copy number variations, it is unlikely to be altered after radiotherapy. Whether gene expression profiling—another method for estimating prognosis in uveal melanoma—is influenced by preceding radiotherapy remains to be established [30, 31].

In summary, post-radiation array CGH for genetic analysis of uveal melanoma seems to be an attractive option to obtain important prognostic information if pre-radiation biopsy was not performed or failed and genetic status is required.

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