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Monosomy 3 by chromogenic in situ hybridization (CISH) in Iranian patients with uveal melanoma

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Abstract

Purpose: The aim of this study was to investigate the rate of monosomy 3 by CISH technique in Iranian patients with uveal melanoma (UM) and its correlation with clinical and histopathological features.
Method: Archival formalin fixed, paraffin-embedded material from 50 patients who had undergone enucleation for large uveal melanomas was obtained. Monosomy of chromosome 3 alteration by chromogenic in situ hybridization (CISH) was investigated. Clinical and histopathological features of tumors were collected.
Results: The patients had a mean age of 56.6 ± 7.6 years. Mean basal diameter and thickness of tumors were 14.1 mm and 10.2 mm, respectively. Four patients (8%) were identified to harbor monosomy of chromosome 3. In the mean follow-up of 5.3 years (range, 3.2–9.5 y), only one case with monosomy 3 died of UM metastasis. The most common type of cellularity was mixed cell (86%).

There was not any statistically significant correlation between monosomy of chromosome 3 and type of cellularity, ciliary body involvement, and largest basal diameter.
Conclusion: The low rate of monosomy chromosome 3 and the consequent low rate of mortality may be indicative of good prognosis in Iranian patients with uveal melanoma.

Keywords: Uveal melanoma; Monosomy of chromosomes 3; CISH

Introduction

The most common primary intraocular tumor, uveal melanoma (UM), has a propensity for hematogenous metastasis that can result in death.[1]

Since the noteworthy report of Prescher et al[2] in 1996, several publications[2–7] investigating the association between the chromosomal abnormalities and UM survival have revealed that the monosomy chromosome 3 is the most frequent karyotypic abnormality in this malignant intraocular tumor, predicting the worse prognosis. Based on the presence or absence of this chromosomal abnormality, two distinct types of tumors have been suggested for this entity: low grade (class I) and high grade (class II) UM.[2–8]

A recent article[9] on molecular classification of UM according to transcriptomic and chromosomal features divided the uveal melanomas into four subgroups based on gene–expression profile and status of chromosomes 3, 6p, and 8p to precisely predict metastatic death: class 1A with minimal aneuploidy, class 1B with 6p gain, class 2A with monosomy 3, and class 2B with monosomy 3 and 8p loss.

Several reports on survival analyses of UM have indicated that monosomy 3 is a strong metastatic risk factor which can lead to decreasing the 5-year survival from 100% to less than

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50%.[2] It has been suggested[10] that this cytogenetic alteration might be the early event that can lead to subsequent increased genomic instability and a higher chance of metastasis due to greater numbers of chromosomal abnormalities (aneuploidy) in tumors with this abnormality.

A variety of DNA-based chromosome testing have been used to detect monosomy 3 in UM.[11]

Between available techniques, chromogenic in situ hybridization (CISH) is promising as a practical, cost-effective, and valid alternative[12] to fluorescent in situ hybridization (FISH). Interpretation of CISH results is not only easy, but its signals do not generally fade over time.

The goal of this study is to investigate the cytogenetic alteration for monosomy of chromosome 3 by CISH technique and its correlation with clinical and histopathological prognostic factors in Iranian patients with UM.

Materials and methods

Patients and tumor samples

Archival formalin fixed, paraffin-embedded material from 50 patients who had undergone enucleation for large ciliary body or choroidal melanoma at a referral center in Tehran, Iran from June 2002 to June 2012 was obtained. Patients with history of radiation, thermotherapy, and resection prior to enucleation were excluded. The study was approved by the Institutional Review Board of Iran University of Medical Sciences.

Clinical and histopathologic data as well as demographic features of each patient were collected from the charts. The largest basal tumor diameter and tumor thickness were measured by B-scan echography.

Follow-up data from the time of diagnosis to the end of the study in June 2012 were completed by reviewing each patient's charts and/or recalling the patients for comprehensive eye examination and systemic evaluation.

Tissue section preparation

Microtome sections 3–5 μm thick were prepared on coated glass slide super frost plus ground edges 72–90 pcs (Thermo scientific) and incubated at 50–60 °C for 2–16 h and at 70 °C on a hot plate. The tissue sections were dewaxed in very pure xylene for 5 min, rehydrated in 100% ethanol for 3 min, and washed 3 times by deionized water. Sections were bleached using hydrogen peroxide.

Melanin bleaching method

Deparaffinized sections were immersed in running and distilled water and rinsed for three times in 5 min time. Subsequently, sections were incubated in warm dilute 3% H_2O_2 in phosphate buffer (0.05 M, pH 7.4) in a staining jar heated in a water bath at 55 °C and washed again in running water for 5 min and in distilled water three times. Finally, sections were rinsed in 1% acetic acid solution for 2 min[13] (Fig. 1).

Chromogenic in situ hybridization (CISH) technique

CISH method was performed on bleached sections using the ZytoDot CISH Implementation Kit protocol (ZytoVision® and ZytoDot ® are trademarks of ZytoVision GmbH) that is recommended to be used with any separately available digoxigenin-labeled ZytoDot CISH probe. The examination was carried out in two days, including pre-treatment (dewaxing, proteolysis, post-fixation) according to the Kit protocol. In day one, the slides were incubated for 10 min at 70 °C temperature, then for 5 min in xylene and 3 min in 100% ethanol, and washed in deionized water. The slides were transferred to the Heat Pretreatment Solution EDTA and incubated for 15 min and then immediately placed into the deionized water. Next, they were incubated in Pepsin Solution for 5 min at room temperature (RT) in a humidity chamber. Finally, the slides were dehydrated using graded alcohols in ascending order. Hybridizations were performed using centromere specific probes to chromosomes 3 (ZytoDot CEN 3 Probe, ZytoVision, Germany). The ZytoDot CEN 3 Probe (PD25) was vortexed and pipetted 10 μl each onto individual samples and denatured the slides at 94–95 °C for 5 min. Then slides were incubated in a humidity chamber and hybridized overnight at 37 °C, e.g. on a hot plate (In situ hybridization system, Hychrome, Euroclone Company, Italy). In day two, Wash Buffer SSC (WB1) was prepared in two staining jars, one at RT, the other heated to 75 °C (depending on the number of slides, the temperature should be increased by 1 °C per slide). PBS/Tween was prepared by adding one tablet of PBS/Tween (WB4) to 1000 ml deionized or distilled water and dissolved. Staining jars were filled with PBS/Tween. Blocking Solution (BS1), Mouse-Anti-DIG (AB1), Anti-Mouse-HRP-Polymer (AB2), Mayer's Hematoxylin Solution, Mounting Solution (alcoholic), DAB Solution, and 3% H_2O_2 were used and prepared (Fig. 2).

Interpretation of results

Visualization of signals was performed using x100 objective, resulting in easily visible signals. In an interphase nucleus of normal cells or cells without aberrations of chromosome 3, the specific signals of hybridization of two chromosomes 3 appear as a DAB brown-colored distinct dot-shaped signal, which can be clearly distinguished from the background counterstained with hematoxylin. A minimum of 100 nuclei were counted. A tumor was identified as monosomic for chromosome 3 if the percentage of nuclei with one hybridization site was greater than 60% of the nuclei counted. In cells with an aneuploidy of chromosome 3, a different signal pattern is visible in interphases.[12]

Retinal tissue adjacent to the tumor was used as normal controls for comparing with the tumor cells.

Statistical analysis

Comparisons of the distributions of clinical and chromosomal variables were performed with the Fisher exact test (for

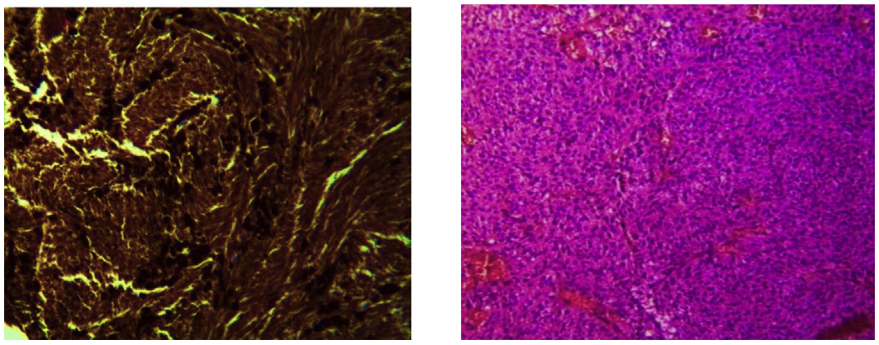

Fig. 1. Deeply pigmented Uveal Melanoma, (left) before and (right) after bleaching using diluted hydrogen peroxide for 2 h at 55 °C. H &E stain (X10).

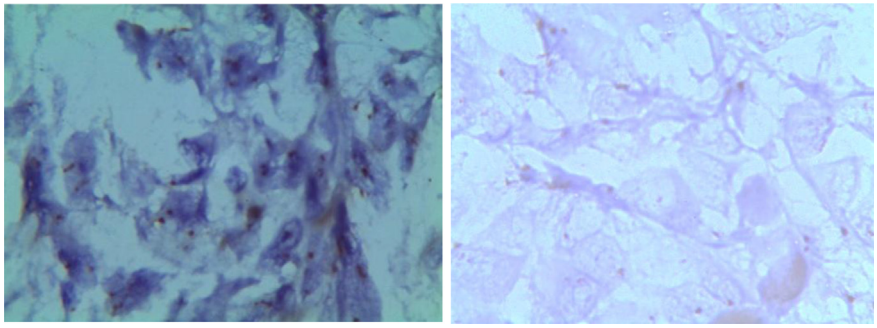

Fig. 2. Chromogenic in situ hybridization (CISH). Left: melanoma hybridized with chromosome 3, showing two copies (Diasomy) in most cells. Right: melanoma hybridized with chromosome 3, showing one copy (monosomy) in most cells (X100).

categorical variables) and the Mann–Whitney test (for continuous variables).

To identify the independent value of the prognostic factors on Disease-Free Survival (DFS), we used a multivariate Cox proportional hazard analysis and the likelihood ratio test. Possible prognostic factors were age at time of diagnosis, cell type (mixed/epithelioid cell), tumor location (ciliary body/choroid), largest tumor diameter, tumor thickness, and monosomy of chromosome 3. Analyses were performed using SPSS version 18 (SPSS, Chicago, IL software), and a P value $\leq$ 0.05 was considered statistically significant.

Results

Of the 50 participants in this study, 22 (44%) were male, and 28 (56%) were female. The mean age at the time of diagnosis was 56.6 years (range, 42–78 years, SD = $\pm$7.6).

The mean and median of the largest basal diameter were 14.1 (SD = $\pm$1.4) and 14.5 mm (range 9–15.8), respectively. The average thickness was 10.2 $\pm$ 1.3 mm (range, 7.2–12.5). Thirty-nine (78%) tumors were located in the choroid, and eleven (22%) tumors showed ciliochoroidal involvement. Cell type was classified as mixed in forty-three (86%) and epithelioid in seven (14%) tumors. None of them was classified as spindle cell type.

Based on the CISH analysis, genomic abnormality of monosomy of chromosome 3 was determined in four (8%) of the 50 specimens. Only one (2%) patient with monosomy of chromosome 3 died of liver metastasis, and three other patients were alive at the end of the study. The patient with liver metastasis was a 62-year-old female with mixed-type choroidal malignant melanoma. The largest diameter of the tumor was 12.80 mm, and the thickness was 11.10 mm.

Univariate analysis of the single possible prognostic factors did not show a statistically significantly higher metastasis incidence rate in patients with potential prognostic factors such as gender, age at time of diagnosis, mixed/epithelioid cell type in the tumor, tumor location, and monosomy of chromosome 3 tumors compared with patients without this chromosomal alteration.

Cytogenetic abnormality (monosomy of chromosome 3) was not correlated with gender, age, ciliary body involvement, basal tumor diameter and thickness, or epithelioid cell type (Table 1).

The mean tumor basal diameter in tumors involving ciliary body was 12.6 $\pm$ 2.03 (SD), which was significantly smaller than the mean value in tumors involving choroid; 14.5 $\pm$ 0.8 (P $<$ 0.0001).

The mean tumor thickness in tumors involving ciliary body was significantly larger than in tumors involving only the choroid, (11.0 $\pm$ 1.30 mm *vs.* 10.0 $\pm$ 1.2 mm; P = 0.019).

The mean follow-up time for all of the patients was 63.7 months (median, 64.2; range, 38.4–115.2). The mean follow-up time for the patients with cytogenetic evidence of monosomy of Chromosome 3 was 45.2 months (median, 45.8; range, 16.8–51.6), and the follow-up time for the only patient who died of metastasis of UM was 16.8 months.

Discussion

To the best of our knowledge, this is the first study on the incidence of monosomy of chromosome 3 in UM in Middle

Table 1
Correlation between monosomy of Ch. 3 and clinical data.

Clinical data		Monosomy of Ch. 3		
		Pos.	Neg.	P*
Gender				0.598[a]
	Male	2	20	
	Female	2	26	
Mean age (y)		62	64	0.327[b]
Cell type				0.536[a]
	Mixed	4	39	
	Epithelioid	0	7	
Mean tumor diameter (mm)		14.2	14.1	0.956[b]
Mean tumor thickness (mm)		10.0	11.0	0.761[b]
Involvement of ciliary body				0.357[a]
	Yes	0	35	
	No	4	11	

[a]Comparison among different subgroups within a chromosome aberration group (*P* calculated by the Fisher exact test).
[b]Comparison of means among different subgroups within a chromosome aberration group, (*P* calculated by Mann–Whitney test).

East. We detected this abnormality in 8% of our cases, but our study failed to show any correlation between monosomy 3 and clinical or histopathological features of tumors. Similarly, Aalto et al[14] did not find any association between clinicopathologic parameters (e.g., cell type, tumor size, and location) and chromosomal alterations.

In contrast, several other studies have shown a correlation between monosomy 3 and more aggressive histopathologic and clinical factors including the largest basal diameter, ciliary body involvement, closed vascular loop, and epithelioid type cellularity.[2,5,7,15]

Because of significant variability in clinical and histopathological parameters and the presence of focal or diffuse heterogeneity[16] of monosomy 3 in UM and different techniques[17] employed to report this abnormality, it has been emphasized that they should be interpreted together to enhance prediction of disease-specific mortality in this tumor.[7]

Although several techniques have been introduced to detect monosomy 3, no consensus has been reported in terms of standard protocol and method between different centers.[18]

Depending on tumor features and cytogenetic techniques used, the detection rate of monosomy 3 ranges from as low as 12% to as high as 65%.[17] The role of monosomy 3 as a major risk factor in UM has been confirmed in different studies, despite diversity in techniques. In our study, the testing of chromosome 3 alteration by CISH technique may not have detected all cases with monosomy 3, making it necessary to confirm the results by other sophisticated cytogenetic tests, such as multiplex ligation-dependent probe amplification (MLPA), which has been suggested by Damato et al,[19] or microsatellite analysis (MSA), which has been advocated by Thomas et al.[20]

As proposed by Sandinha et al,[12] we reported a tumor as monosomic for chromosome 3 if the percentage of nuclei with one hybridization site was greater than 60% of the nuclei counted. Therefore, the lower detection rate in our study might be due to a relatively high threshold of 60%, which was considered to define monosomy 3 status by CISH technique.

The low incidence of monosomy 3 along with 2% mortality rate in our study are in accordance with published data from other Asian countries.[21,22]

Epidemiologic data on population-based incidence of UM by Hu et al[23] showed that the relative risk of UM was 1.2 for Asian and Pacific Islander patients, 5.4 for Hispanic patients, and 19.2 for non-Hispanic white patients as compared with black patients.

They calculated that the annual age-adjusted incidence rate of UM per million population was 0.31 for African-Americans, 0.38 for Asians, 1.67 for Hispanics, and 6.02 for non-Hispanic Caucasians.

The same variability have been suggested in biological features and clinical presentation by some authors between different ethnic groups.[24–26]

These facts may be indicative that the role of race as an independent prognostic factor should also be investigated in detail in UM.

Our study had some limitations. We included small sample size of patients from one referral center, which is not representative of the entire Iranian population. A multivariate analysis of different clinical and cytogenetic prognostic factors was impossible due to the small sample size, and additional studies with a larger sample size to investigate the correlation of monosomy 3 with metastasis-related death will be required. We did not perform any other method to validate the results of CISH technique. The lower rate of monosomy 3, might be due to sampling error and tumor heterogeneity.

Furthermore, despite choosing the best method of bleaching for lessening the interference with hybridization, bleaching tissue sections in heavily pigmented specimens in our study inevitably affected the quality of CISH sections. Interpretation difficulties also were encountered with overlapping nuclei, particulate debris on slides, and also with highly pigmented tissues that were not completely depigmented due to signal interference by residual pigments.

In conclusion, our results demonstrated the less frequent events of monosomy chromosome 3, epithelioid cellularity, and lower rate of UM-related death in Iranian patients, which is different from previously published reports in Caucasian populations harboring this tumor.

The value of our report is enhanced by the fact that the incidence of this cytogenetic abnormality in Iranian patients is the lowest rate that has been reported so far. Further studies are warranted to evaluate the different aspects of chromosomal abnormalities and clinical characteristics in UM as well as their correlation among different non-Caucasian ethnic groups.

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