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Role of molecular and genetic factors in survival from uveal melanoma

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Aim — to analyze survival rates in uveal melanoma (UM) patients and establish correlations with chromosome 3 monosomy, chromosome 1p deletion, and *RASSF1A* methylation. **Material and methods.** Methylation-specific PCR analysis was performed in 104 patients with histologically verified UM. **Results.** A statistically significant correlation has been found between chromosome 3 monosomy, on the one hand, and mixed/epithelioid cell melanomas and ciliary body involvement, on the other. As for chromosome 1p deletion, it has demonstrated association with extrabulbar tumor growth. We have also calculated 5-year survival and mortality rates in «large» UMs and their relationship with chromosome 3 monosomy, chromosome 1p deletion, and *RASSF1A* methylation. **Conclusion.** Chromosome 3 monosomy is associated with lower survival rates, while *RASSF1A* methylation — with a better prognosis. A combination of molecular and genetic changes (particularly, chromosome 3 monosomy and chromosome 1p deletion) also leads to reduced survival in UM patients.

Keywords: *uveal melanoma, chromosome 3 monosomy, chromosome 1p deletion, RASSF1A methylation, survival rate.*

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Uveal melanoma (UM) – the most widespread primary intraocular malignant tumor among adult population [1, 2]. There are also individual cases of UM reported in children and teenagers [3, 4]. About 50% of patients with UM die from metastatic disease. This proportion remained constant during the last century [5]. In 1996 G. Prescher and co-authors [6] revealed the significant correlation between the full loss (monosomy) of chromosome 3 in the tumor and mortality of patients from metastatic disease. Later on this has been confirmed by the other scientists independent of the methods used for genotyping for chromosome 3 status identification [7–11]. Duplication of chromosome 8q enhances tumor progression in case of monosomy 3, but not in case of disomy of chromosome 3. In UM the chromosome rearrangements in the short arm of chromosome 1 (chromosome 1p deletion) is observed in one third of patients and are similar in structure with the pathology seen in skin melanoma [12]. It's noted that the presence of rearrangements in short arm of chromosome 1 in melanomas are the signs of tumor progression. T. Hausler and co-authors [13] have found statistically significant (p=0,0001) correlation between deletion of short arm of chromosome 1 and monosomy 3.

In the research on the gene expression profile (GEP) it is shown that there are two classes of UM associated with the status of chromosome 3 and vital prognosis [14, 15]. According to the data by L. Worley and co-authors [16], GEP can be considered as more exact criterion for the prognosis of metastasis compared to chromosome 3 monosomy when comparative genomic hybridization or fluorescent in situ hybridization is used as an identification method. One of the studied genes is *RASSF1A*, specific for skin melanoma. Methylation of *RASSF1A* is shown also for cellular lines of UM (91%) and in the sample of 39 primary UM the prevalence is 50% [17]. *RASSF1A* is located in the locus p21.3 chromosome 3, the most frequently damaged chromosome in UM cells and is a regulator of cell cycle; the decrease in its expression damages the control of cell cycle in phase G1/S, it is the suppressor of cycline D, the hyperexpression of which leads to functional inactivation of RB that causes progression of UM [18].

The profound correlation of UM molecular-genetic profile with the metastatic disease contributes to the development of this diagnostic method and research shows that the majority of patients prefer to know the risk of metastasis occurrence independent of efficacy and accessibility of treatment methods [19–21]. The possibility to perform adjuvant therapy in UM represents practical significance of molecular-genetic analysis, since this analysis could be done in patients with high risk of metastasis occurrence [19]. Due to the importance of molecular-genetic aberrations identification for the prognosis of UM we have set up the aim – to analyze the survival in the group of UM patients and its correlation with such disorders as chromosome 3 monosomy, deletion of short arm of chromosome 1 and methylation of gene *RASSF1A*.

Materials and methods

Clinical data. 104 UM patients treated during the period of 2005–2007 have been examined. The age varied

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from 22 to 84 years (mean age 53,7 ± 12,2 years). There were 66 women (63,4%) and 38 men (36,6%). The follow-up period comprised 41-84 months (60,9±8,8 months).

101 patients (97,1%) patients have had enucleation, 3 patients (2,9%) had tumor local excision performed on indication. In 4 (3,9%) patients the enucleation was preceded by organpreserving treatment (brachytherapy course with the use of Ru/Rh plaques).

According to localization melanoma of ciliochoroidal zone was diagnosed in 26 patients (25%), melanoma of choroid in 67 (64,4%), iris melanoma in 4 (3,85%) and iridociliochoroidal zone melanoma in 7 patients (6,7%).

The tissue samples. After enucleation of the damaged eye the biopsied material of tumor and relatively non-damaged choroid, as well as the sample of peripheral blood (preservative – 0,5 M EDTA solution) have been kept at -20°. Cytologic samples were used for the investigation of material from fine needle aspiration biopsy.

The genomic DNA from the tumor samples, from relatively intact choroid and peripheral blood has been isolated with proteinase K with the following phenol-chloroform extraction. During the isolation of DNA from cytologic samples the material from the slides was treated by lysing buffer, containing proteinase K and the formed lysate was used as a matrix for polymerase chain reaction (PCR).

The loss of heterozygosity in chromosome regions 1p26, p31.3, 3p25.3, 3p21.3, 3p14.2, 3q12, 3q26.3, 3q28 has been identified using highly polymorphic markers D1S243, D1S2145, D1S1635, D1S407, D1S3669, D1S438, D3S1038, D3S1317, D3S1568, D3S966, D3S1300, D3S1234, D3S2459, 16xTG_3q26.31, D3S3520, D3S2398. For the control the DNA of leucocytes from the peripheral blood was used.

The method of methylsensitive PCR was used for the identification of methylation of CpG-islands of promoter fields of genes. As a matrix for PCR the DNA of melanoma cells was used, which was hydrolyzed before that by restrictases HpaII (for the gene *RASSF1A*).

Screening in patients with UM. The screening has been performed according to the law №135 of Ministry of Health Russian Federation from 19.04.1999 that is once in three months during the first year, then once in six months during the second year, later on once a year in Adult Outpatient-consultation Department of Moscow Helmholtz Research Institute of Eye Diseases. Patients who for various reasons could not come to the control examination have reported by phone, fax and through the relatives passing by the actual record of consultation from local oncologist and ophthalmologist. Part of the data has been claimed by special inquiry to the local Health Department at the patient's residence.

The statistical analyses has been performed by Fisher's exact criterion and its significance. To study the survival of patients after the treatment the statistical method of multiple estimates by Kaplan-Meyer, and for the estimation of the difference between two samples –Wilcoxon criteria (Mann-Witney) and regressive Cox model. To analyze the information all data have been put into two groups: censored (non-finished) where the outcome has not come at the time of finalization of the study (patients are alive) or patients dropped out of the study (the included reason is the death from the other cause) and non-censored (finalized) where the death of patient was due to metastatic disease. The data analyses has been performed using Microsoft Excel and Statistica 10.1.

Results and Discussion

From the period of October 2005 to November 2007 in the Department of Ocular Oncology and Radiology, Moscow Helmholtz Research Institute of Eye Diseases, 101 enucleations were performed and 3 local excisions of UM with the following pathohistologic and molecular-genetic analysis in all studied cases. All cases have been followed up for a long-term period of 5 years and more. According to the results of the molecular-genetic analysis all patients have been divided into the three groups: first group – patients with chromosome 3 monosomy (n=45; 43,4%), 2nd group – patients with deletion of the short arm of chromosome 1(n=30, 28,8%) and 3rd group with methylation of *RASSF1A* gene (n=23, 22,1%). The comparative group was represented by patients without the aberrations studied. In 11,5% of cases (n=12) chromosome 3 monosomy and short arm of chromosome 1 deletion was observed in patients. In 5,8% of cases (n=6) both chromosome 3 monosomy and methylation of gene *RASSF1A* was observed. The combination of chromosome 3 monosomy and methylation of gene *RASSF1A* has been found in 9 (8,7%) out of 104 studied patients. Finally combination of 3 molecular-genetic aberrations has been found in 2 (1,9%) patients. In 29 patients (27,9%) none of these aberrations were found.

For each group the clinical and pathomorphologic characteristics were outlined (**see the Table**). Statistically significant ($p<0,05$) correlations were found in patients from the chromosome 3 monosomy group and corresponding histologic tumor types, especially that for this group the prevalence of prognostically least beneficial types, i.e. mixed type and epithelioid type (77,8%). The correlation analyses of histologic UM types with the tumors of 1p group and *RASSF1A* group did not show the significant differences for UM types. In the group with chromosome 3 monosomy there also has been found the association with the involvement of ciliary body into the tumor process which is considered to be a negative factor aggravating the vital prognosis [22]. Thus in this group we observed more than half of patients (51,1%) had ciliochoroid and iridociliochoroid UM localization, which seems of importance comparing to groups with 1p, *RASSF1A* and general group of patients with dominance of tumors of choroid localization non-involving ciliary body (p=0,038). The group of patients with short arm of

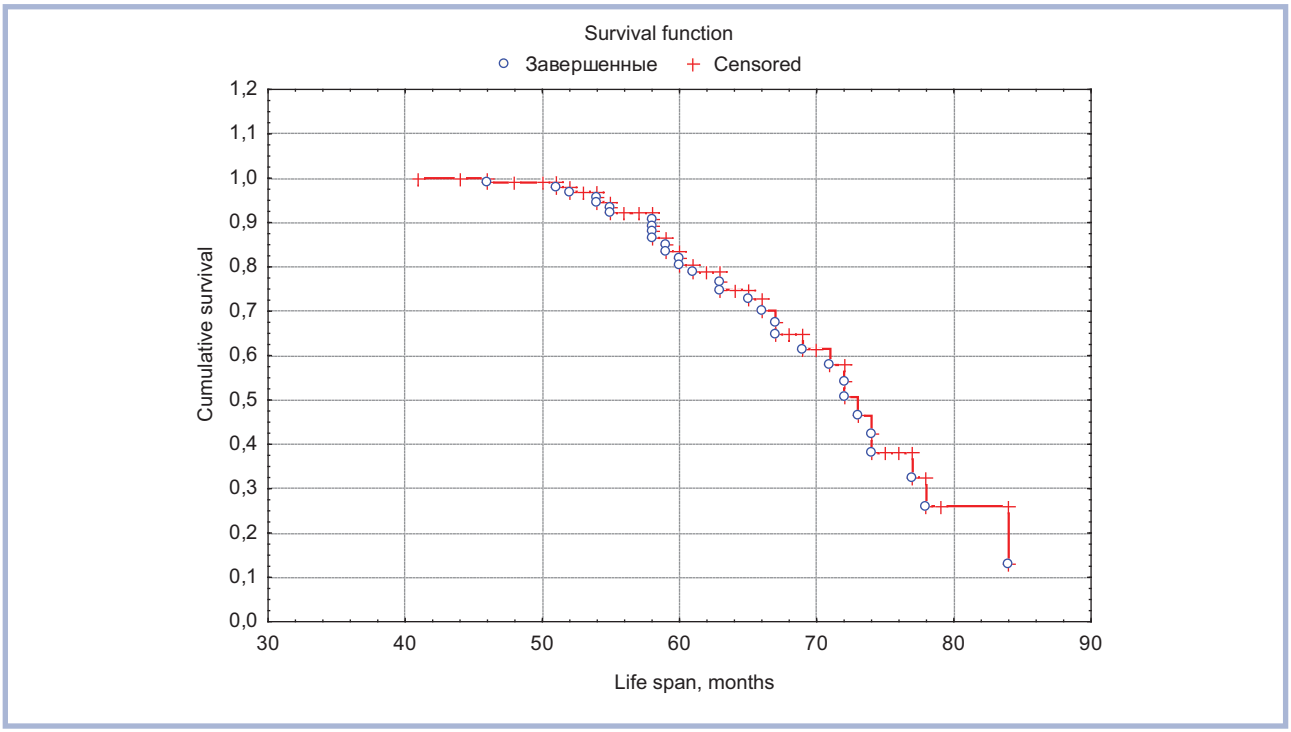


Fig.1. **The general survival in oncology patients.**

chromosome 1 deletion was found to have a significant association with the extrabulbar tumor growth which is one the most adverse prognostic factors which amplifies the probability of metastatic disease [23]. According to our data the survival in the group with short arm of chromosome 1 deletion did not differ from the general survival therefore this association could be accidental.

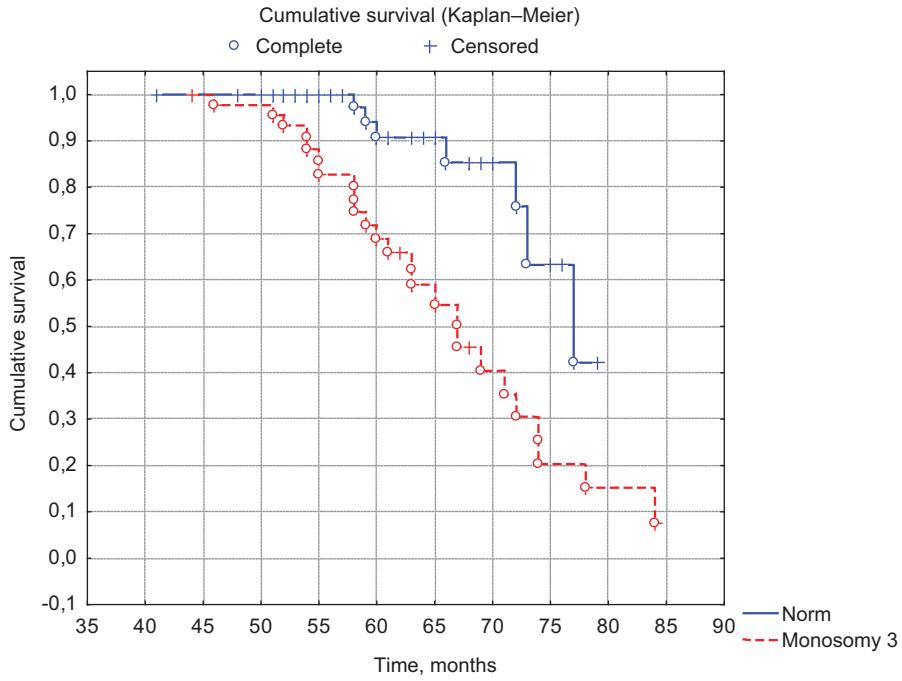
Mortality from the metastatic disease was 30,8% (n=32) (**Fig.1**). 69,2% of general number of patients was represented as alive during the examination (n=65, 62,5%) and dead due to other causes (n=7, 6,7%). The mean follow- up period in the general group comprised 60,1±9,3 months, the mean life span from radical surgical treatment to death due to metastatic disease – 62,9±10,1 months. It should be noted that the research comprised patients with "large" UM (mean height of the tumor – 9,3±2,7 mm, basal diameter 15,7±3,6 mm). Thus our data on general survival correlates to the other authors' data specialized on this subject [24].

Also of a special interest was the study of cumulative number of survived in the group with chromosome 3 monosomy (n=47) versus the control group (n=57) (**Fig.2,a**). The analyses shown that the mortality from the metastatic disease was higher than the survival (53,2% vs 46,8%, p=0,002) in the chromosome 3 monosomy group, which demonstrates special role of chromosome 3 monosomy in the UM patients' survival. Similar data is presented by foreign authors [13,15,20]. The survival in the control group was 87,7%, the mortality – 12,3 % which is 4 times lower than in chromosome 3 monosomy group. Therefore we confirm the correlation of chromosome 3 monosomy with the decreased survival in UM.

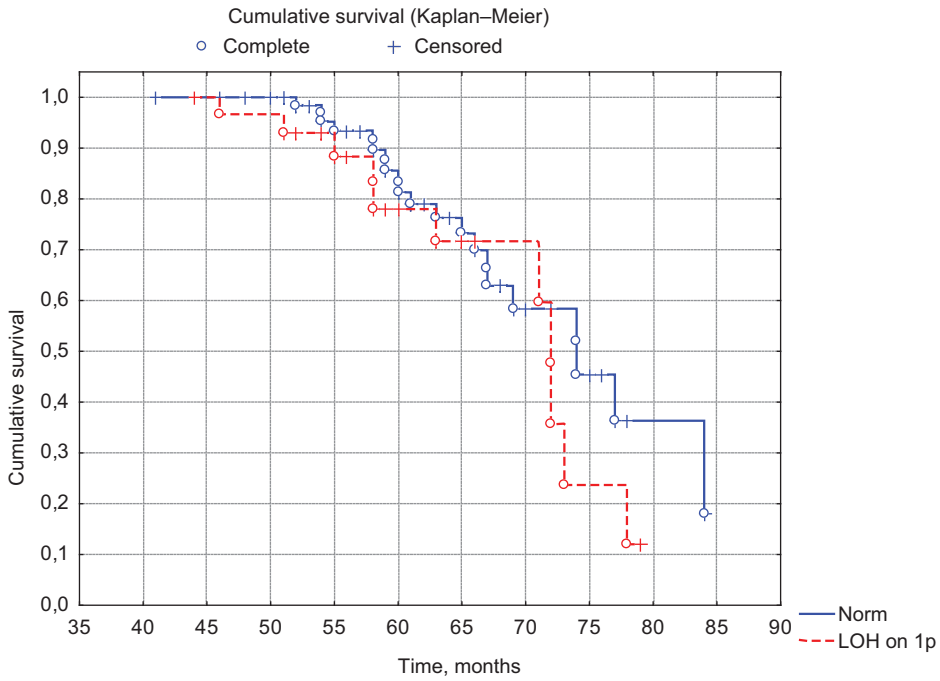
The survival analysis showed that the mortality in group 1p (short arm of chromosome 1 deletion group, n=30) comprised 36,7% and 28,4% in the control group (n=74) accordingly (**see Fig.2,b**). The survival difference in the two groups is statistically non-significant (p=0,064). Similar data was received during the comparison of patients with methylation of *RASSF1A* (n=23) where the mortality was 26,1% and 32,1% mortality in the control group (n=81) (**Fig.3**). Here it should be noted that this difference was statistically significant (p=0,037 according to Cox regression model) compared to the group with short arm of chromosome 1 deletion, and thus could demonstrate more favourable course of tumor status in tumors with methylated gene *RASSF1A*.

In the group of patients with the combination of chromosome 3 monosomy and short arm of chromosome 1 deletion (n=12) the mortality was 75% which is significantly higher than the general mortality (p=0,001). We revealed the mortality in 2/3 of cases, 67% (p=0,03) in patients with combination of chromosome 3 monosomy and methylation of gene *RASSF1A* (n=6) and the mortality of 22,2% (p=0,068) in patients with combination of short arm of chromosome 1 deletion and methylation of gene *RASSF1A*. Out of 2 patients who had all three molecular-genetic aberrations one has died from a metastatic disease in 51 months after the performed treatment.

Therefore the deletion of short arm of chromosome 1 has not significantly influenced the survival of UM pa-



a



b

Fig. 2 **. The association of the patients' survival with the chromosome status.**

a – chromosome monosomy 3; b – deletion of short arm of chromosome 1.

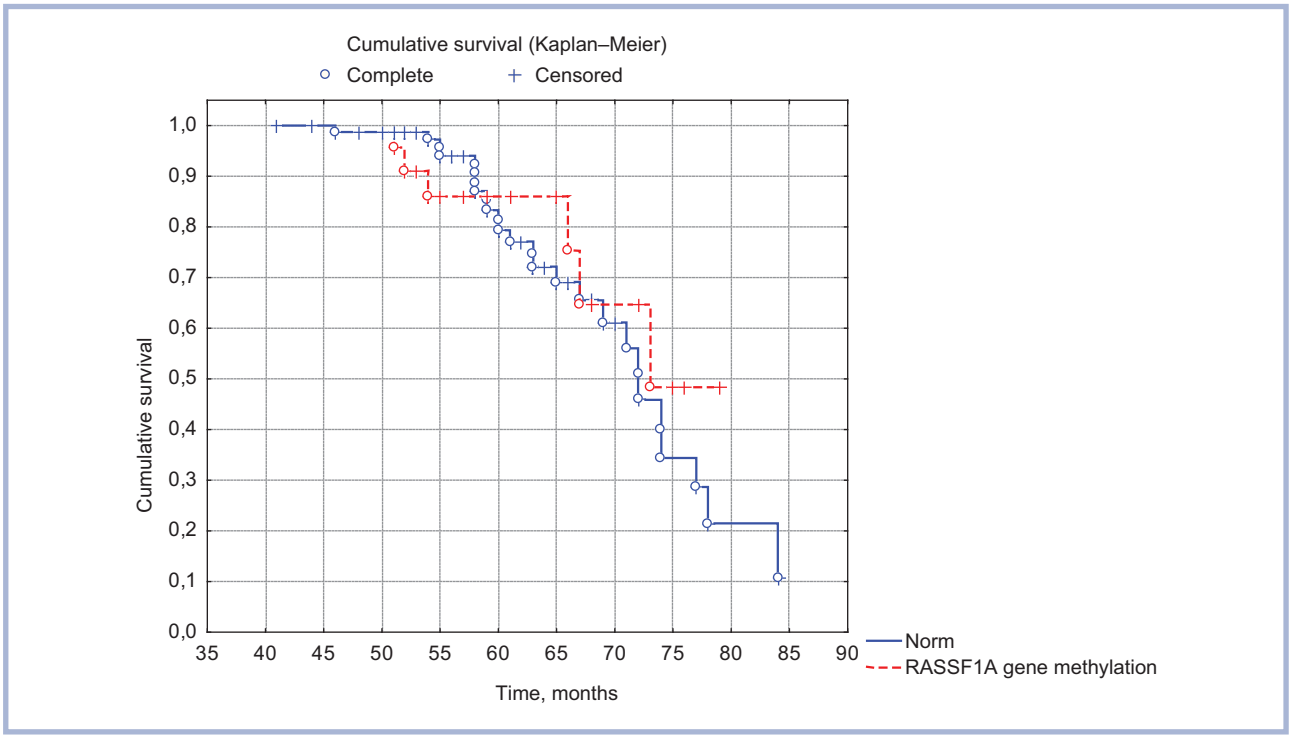


Fig. 3. **The association of the patients' survival with methylation of gene *RASS1A*.**

Clinical and morphological features of patients

Characteristics	Groups			
	M3 (*n*=45)	1p (*n*=30)	*RASSF1A* (*n*=23)	Comparison (n=29)
Age at the time of surgery (years)	55±9,8	53±9,2	57±10,1	53±8,9
Sex:				
male	18 (40%)	10 (33,3%)	10 (43,5%)	6 (20,7%)
female	27 (60%)	20 (66,7%)	13 (56,5%)	23 (79,3%)
Involvement of ciliary body:				6 (20,7%)
with involvement	23 (51,1%)*	11 (36,7%)	5 (21,7%)	23 (79,3%)
without involvement	22 (48,9%)*	19 (63,3%)	18 (78,3%)	
Pigmented	41 (91,1%)	28 (93,3%)	22 (95,6%)	19 (65,5%)*
Non-pigmented	4 (8,9%)	2 (6,7%)	1 (4,4%)	10 (34,5%)*
Hemophthalmia	8 (17,8%)	13 (43,3%)	8 (34,8%)	11 (37,9%)
Retinal detachment	28 (62,2%)	19 (63,3%)	16 (69,5%)	21 (72,4%)
Subretinal exudate	8 (17,8%)	9 (30%)	6 (26,1%)	5 (17,2%)
Visible tumor vessels	23 (51,1%)	17 (56,7%)	11 (47,8%)	15 (51,7%)
Extrabulbar growth	5 (11,1%)	5 (16,7%)*	2 (8,7%)	3 (10,3%)
Burdened familial history in terms of oncopathology	9 (20%)	5 (16,7%)	4 (17,4%)	7 (24,1%)
Tumor height (mm)	9,6±2,7	9,3±2,4	8,8±2,1	9,1±2,2
Tumor basal diameter (mm)	16,3±3,7	16,4±3,9	15,8±3,5	15,1±3,2
Type of tumor:				
spindle cell	10 (22,2%)*	13 (43,3%)	12 (52,1%)	19 (65,5%)*
mixed	23 (51,1%)*	13 (43,3%)	7 (30,4%)	6 (20,7%)*
epithelioid	12 (26,7%)*	4 (13,4%)	4 (17,4%)	4 (13,8%)*

Note: * — statistically significant ($p<0,05$).

tients and methylation of gene *RASSF1A* is associated with more positive vital prognosis. The combination of several molecular-genetic disorders significantly decreased patients' survival.

Conclusions

1. The distribution of molecular-genetic disorders such as chromosome 3 monosomy, deletion of short arm of chromosome 1 and methylation of gene *RASSF1A* and their correlation with clinical and morphologic parameters of UM has been studied.

2. The statistically significant correlation of chromosome 3 monosomy with both mixed- and epithelioid-cell type of UM, involvement of ciliary body into the process, as well as association of short arm of chromosome 1 deletion with extrabulbar tumor growth has been shown.

3. The 5-year survival and mortality as a result of metastatic disease in patients with "large" UM has been studied.

4. The statistically significant association between chromosome 3 monosomy and decreased patients' survival as well as between methylation of gene *RASSF1A* and improvement of vitality prognosis have been demonstrated.

5. The combination of two or more molecular-genetic disorders worsens the UM patients' survival.

6. The found correlations could be used for the prognosis of the UM course in patients while performing the enucleation, tumor local excision, fine needle aspiration biopsy, as well as performance in the future of adjuvant therapy for UM in patients with high risk of metastasis development.

Author contributions:
Study conception and design: S.S., D.Z.
Data collection and management: A.T., N.S., D.Z.
Statistical data analysis: A.A., A.T.
Text: A.T., N.S.
Editing and review: S.S., A.A.

There is no conflict of interest.

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