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Nuclear Pseudoinclusions and Intranuclear Grooves Have an Important Impact on the Long-term Survival of Patients With Uveal Melanoma

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Abstract. *Aim: The evaluation of the prognostic role of nuclear pseudoinclusions (NPIs) and nuclear grooves (NGs) in UM. Patients and Methods: We examined the presence of NPIs and NGs in hematoxylin and eosin-stained tissue sections from 164 removed eyeballs with uveal melanoma (UM) and analyzed statistical relationships with clinical and pathological parameters and the long-term survival rate. Results: We observed NPIs in 38% and NG in 21% of all UM. The presence of NPIs was significantly positively correlated with epithelioid type, marked pleomorphism, and the presence of multinucleated giant cells, macro-nucleoli and multiple nucleoli. Patients with UM with NPIs had a significantly reduced overall survival rate ($p<0.0001$). The presence of NGs was significantly inversely correlated with marked pleomorphism, and the presence of multinucleated giant cells, macro-nucleoli and multiple nucleoli. Kaplan–Meier analysis demonstrated significantly better overall ($p<0.01$) and disease-free ($p<0.05$) survival rates for patients with NGs. Conclusion: The obtained results suggest that the presence of NGs in UM is associated with a better prognosis, as opposed to the presence of NPIs, which means the prognosis is worse.*

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Uveal melanoma (UM) is the most frequently occurring primary intraocular tumor in adult patients. Its prevalence varies depending on race and geographic latitude, and is highest amongst Caucasian (98% of all cases) and those at higher latitudes. In Mediterranean countries, this means two new cases per 1,000,000 inhabitants per year, whilst in Scandinavian countries this number is higher at 8-11. In the United States of America, there are approximately four new cases per 1,000,000 people per year on average (1-5).

Melanoma involves various parts of the uvea with varied frequency: 4-6% of cases are located in the iris, 6-9% in the ciliary body, with the choroid being the most frequent location at 85-90% (6, 7). The prognosis in UM depends on multiple factors – one of them being the size of the tumor (the largest diameter at the base and height). Larger tumors are characterized by poorer survival rates. Data analysis presented by Shields *et al*., comprising 8033 cases, showed that tumor increasing by 1 mm, increases the risk of metastases within 10 years by 5%. According to the largest base diameter, they divided the tumors into three categories: Small (0-3 mm), medium (3.1-8.0 mm) and large (>8 mm) (8). In these groups, 5-, 10- and 20-year mortality was 6%, 12% and 20%, 14%, 26% and 37%, and 35%, 49% and 67%, respectively (8, 9).

Another factor which negatively affects prognosis is the involvement of the ciliary body by UM (6, 8).

A wide range of histological factors have been linked to metastasis formation, such as the epithelioid cell type, intrascleral and extrascleral extension, high mitotic activity, optic nerve extension, vasculogenic mimicry patterns containing arcs with branching, closed vascular loops and vascular networks, as well as immune infiltration with an increased

number of lymphocytes (mainly T-cells) and macrophages, which are associated with a poorer prognosis (6, 10-13).

Chromosome 3 monosomy, two or more copies of 1q, 6p and 8q, losses of 1p, 6q and 8p, and inactivation of *BAP1* gene encoding BRCA1-associated protein 1 (located on chromosome 3) are related to a high risk of UM metastasis. In contrast, mutation of eukaryotic translation initiation factor 1A X-linked (*EIF1AX*) is associated with an excellent survival outcome (6, 7, 14, 15).

Local control of the tumor is very high (86-95%) and is obtained as a result of the application of various conservative treatment methods, such as brachytherapy, proton therapy, trans-pupillary thermotherapy, tumor endo- or exoresection, and a combination of the above methods. In the case of very large tumors, where the diameter of the base is larger than 20 mm, or the height exceeds 12 mm, and if the tumor significantly involves the optic disc, the best treatment method still remains the enucleation of the affected eye (16).

A high mortality rate, even reaching 50% on account of systemic dissemination of the cancer with lack of an effective treatment method, still remains a significant problem (13). In more than 90% of cases, metastases are located in the liver, in spite of the positive effects of local treatment. On account of these factors, the search for new prognostic and therapeutic factors which may assist in the treatment of patients with UM remains an important issue.

In various cancer types, pathologists look for cancer cell-specific morphological properties (including cellular nucleus) and attempt to evaluate these, as they may help in improving diagnosis and determining the prognosis. Among others, the shape of the nuclear envelope and intranuclear structures are taken into consideration. In this category of properties, the analysis frequently concerns nuclear pseudoinclusions (NPIs) and nuclear grooves (NGs). The presence of NPIs and NGs in the cellular nuclei is evaluated during cytological and histopathological assessments, most frequently after hematoxylin and eosin (H&E) staining.

NPIs are vacuoles in a cellular nucleus, limited by the nuclear membrane – they are formed by an intussusception of cytoplasm inside the nucleus. They do not have direct contact with the nucleoplasm. H&E staining allows one to see the eosinophilic material resembling cytoplasm in color or slightly paler shapes inside them. NPIs must be differentiated from true nuclear inclusions and pseudo-pseudoinclusions ('bubbly' nuclei).

The presence of NPIs is described in various types of tissues, including cancer tissues (most frequently in papillary thyroid carcinoma, meningioma, ductal hyperplasia of the breast, pituitary adenoma and lung adenocarcinoma), yet their etiopathogenetic and prognostic significance in oncology remains unclear (17-24).

Another feature of cellular nuclei, occurring in various tissues, are NGs, described as 'coffee bean' shape nuclei. This is the property of cellular nuclei consisting of clear longitudinal invaginations of the nuclear envelope bilayer observed after H&E staining. NGs may be indicative of the cancerous character of a lesion. NGs have been described in various types of cancer, such as papillary thyroid carcinoma (100% of cases), ependymoma (81% of cases), primary breast carcinomas (78% of cases), metastatic breast carcinoma (90% of cases) and in adult granulosa cell tumor (90% of cases) (25-27). However, NGs are also found in a normal cervical smear and benign bronchioalveolar lavage (28).

The presence of NPIs and NGs in the cells of examined tissues may also suggest their malignant character, as is the case in papillary thyroid carcinoma. Yet some authors call into question their significance in the differentiation between malignant and benign lesions (28, 29).

The available literature on the subject contains some individual reports on the presence of NPIs and NGs in UM. However, in none of them is there a relationship between their presence and the clinical picture of the tumor, its histopathological properties and patient survival rates. Therefore, we decided to analyze the presence of NPIs and NGs and their relationships with the above features (30, 31). To our knowledge, our report is the first to characterize the above issue.

Patients and Methods

The study group consisted of 164 patients with UM treated by primary enucleation at the Department of Ophthalmology and Ocular Oncology, diagnosed between 2002-2011. Patients were enrolled in the study based on the availability of their medical records and tissue specimens, which included paraffin blocks and histology slides. Comprehensive clinical data were retrieved from the archived medical records, and details of diagnostic and therapeutic procedures performed were sourced from the Ocular Oncology Outpatient Clinic, University Hospital. The Authors declare that this investigation was carried out following the rules of the Declaration of Helsinki of 1975 (revised in 2013) and this study was reviewed and approved by the Ethics Committee of the Jagiellonian University, Krakow, Poland (decision no. 122.6120.58.216), and the Wroclaw Medical University, Wroclaw, Poland (decision no. KB-500/2017).

The records were reviewed for clinical and pathological data including age, sex, affected eye, largest basal diameter and height of the tumor, tumor staging (pT and American Joint Committee on Cancer (AJCC) prognostic stage group (32), tumor location relative to the equator, ciliary body involvement, clinical tumor pigmentation and shape, concomitant glaucoma/retinal detachment, histological subtype, scleral/optic nerve infiltration, extraocular extension, as well as tumor necrosis. Additionally, detailed histological parameters, such as mitotic rate, presence of tumor-infiltrating lymphocytes, characteristics of nucleoli (presence, size and number), multinucleated giant cells and hemorrhage, as well as tumor cell pigmentation level were considered (32-34).

The largest basal diameter and height of the tumor were described in line with the AJCC guidelines (32) and staged according to the Collaborative Ocular Melanoma Study (COMS) (32-34).

Table I. *Summary statistics for the relationship between nuclear pseudoinclusions and intranuclear grooves in uveal melanoma cells and clinical parameters.*

		Nuclear pseudoinclusions			Nuclear grooves		
Clinical parameter		Negative	Present	*p*-Value	Negative	Positive	*p*-Value
Frequency, n (%)	Overall	101 (62%)	63 (38%)	-	129 (79%)	35 (21%)	-
Age, years	Mean (range)	60 (51-66)	61 (54-68)	0.38[a]	61 (54-68)	58 (50-62)	**0.020**[a]
Gender, n (%)	Female	52 (51%)	32 (51%)	>0.99[b]	67 (52%)	17 (49%)	0.85[b]
	Male	49 (49%)	31 (49%)		62 (48%)	18 (51%)	
Eye, n (%)	Right	49 (49%)	29 (46%)	0.87[b]	63 (49%)	15 (43%)	0.57[b]
	Left	52 (51%)	34 (54%)		66 (51%)	20 (57%)	
Largest basal tumor diameter, n (%)*	9-12 mm	11 (11%)	2 (3%)	0.17[c]	11 (9%)	2 (6%)	0.32[c]
	>12-15 mm	18 (18%)	9 (14%)		18 (14%)	9 (26%)	
	>15-18 mm	21 (21%)	20 (32%)		35 (27%)	6 (17%)	
	>18 mm	51 (50%)	32 (51%)		65 (50%)	18 (51%)	
Largest basal tumor diameter, n (%)*	<10 mm	2 (2%)	0 (0%)	0.51[c]	2 (2%)	0 (0%)	0.40[c]
	10-<16 mm	31 (31%)	16 (25%)		34 (26%)	13 (37%)	
	≥16 mm	68 (67%)	47 (75%)		93 (72%)	22 (63%)	
Greatest tumor height, n (%)*	≤3 mm	1 (1%)	0 (0%)	0.91[c]	1 (1%)	0 (0%)	0.12[c]
	>3-6 mm	10 (10%)	5 (8%)		12 (9%)	3 (9%)	
	>6-9 mm	27 (27%)	16 (25%)		33 (26%)	10 (29%)	
	>9-12 mm	36 (36%)	22 (35%)		40 (31%)	18 (51%)	
	>12-15 mm	20 (20%)	17 (27%)		34 (26%)	3 (9%)	
	>15 mm	7 (7%)	3 (5%)		9 (7%)	1 (3%)	
Greatest tumor height, n (%)**	<3 mm	1 (1%)	0 (0%)	0.65[c]	1 (1%)	0 (0%)	0.49[c]
	3-<8 mm	18 (18%)	15 (24%)		24 (19%)	9 (26%)	
	≥8 mm	82 (81%)	48 (76%)		104 (81%)	26 (74%)	
Primary tumor (pT), n (%)	2	11 (11%)	2 (3%)	0.19[c]	11 (9%)	2 (6%)	0.24[c]
	3	29 (29%)	22 (35%)		36 (28%)	15 (43%)	
	4	61 (60%)	39 (62%)		82 (64%)	18 (51%)	
Stage, n (%)	IIA	9 (9%)	1 (2%)	0.43[c]	8 (6%)	2 (6%)	0.86[c]
	IIB	27 (27%)	17 (27%)		33 (26%)	11 (31%)	
	IIIA	32 (32%)	23 (37%)		42 (33%)	13 (37%)	
	IIIB	26 (26%)	18 (29%)		37 (29%)	7 (20%)	
	IIIC	7 (7%)	4 (6%)		9 (7%)	2 (6%)	
Ciliary body involvement, n (%)	Not involved	72 (71%)	37 (59%)	0.13[b]	84 (65%)	25 (71%)	0.55[b]
	Involved	29 (29%)	26 (41%)		45 (35%)	10 (29%)	
Degree of pigmentation, n (%)	Amelanotic	19 (20%)	7 (12%)	0.36[c]	17 (14%)	9 (27%)	0.057[c]
	Mildly pigmented	38 (40%)	23 (39%)		46 (38%)	15 (45%)	
	Heavily pigmented	38 (40%)	29 (49%)		58 (48%)	9 (27%)	
Shape, n (%)	Domed	50 (50%)	35 (57%)	0.422	64 (51%)	21 (60%)	0.35[b]
	Mushroom	50 (50%)	26 (43%)		62 (49%)	14 (40%)	
Retinal detachment, n (%)	No	17 (17%)	14 (22%)	0.42[b]	25 (19%)	6 (17%)	>0.99[b]
	Coexistence	84 (83%)	49 (78 %)		104 (81%)	29 (83%)	
Glaucoma, n (%)	No	83 (83%)	57 (90%)	0.25[b]	109 (85%)	31 (89%)	0.79[b]
	Coexistence	17 (17%)	6 (10%)		19 (15%)	4 (11%)	

*American Joint Committee on Cancer (32). **Collaborative Ocular Melanoma Study (32-34). [a]Wilcoxon two-sample test; [b]Fisher's exact test; [c]chi-square test. Statistically significant results ($p<0.05$) are in bold text.

Evaluation of NPIs and NGs. The presence of NPIs and NGs was evaluated in 164 H&E sections of primary UM. NPIs were defined as intranuclear vacuoles with the constituents of a color and morphological structure similar to that of the surrounding cytoplasm or paler. NGs were defined as longitudinal invaginations of the nuclear envelope. The presence of any NPIs or NGs in tumor cells finally categorized a case as positive.

Statistical analysis. Statistical analysis was performed using the R language and the survminer tool (available online: https://www.r-project.org/) (35, 36).

Overall survival (OS) was defined as the time period from the date of UM diagnosis until death date or the last follow-up and disease-free survival (DFS) as the time from finishing UM treatment until metastasis or the last follow-up. In order to determine the OS

Table II. *Summary of statistics for the relationship between nuclear pseudoinclusions and intranuclear grooves in uveal melanoma cells and histopathological parameters.*

Histopathological parameter		Nuclear pseudoinclusions, n (%)			Nuclear grooves, n (%)		
		Negative	Present	*p*-Value	Negative	Positive	*p*-Value
Frequency	Overall	101 (62%)	63 (38%)		129 (79%)	35 (21%)	
Histological subtype	Spindle-cell	25 (25%)	4 (6%)	**0.003**[a]	19 (15%)	10 (29%)	0.079[a]
	Mixed and epithelioid cell	76 (75%)	59 (84%)		110 (85%)	25 (71%)	
Mitotic rate	0-4	66 (66%)	41 (65%)	>0.99[b]	80 (62%)	27 (77%)	0.11[b]
	5-31	34 (34%)	22 (35%)		48 (38%)	8 (23%)	
Extraocular extension	No	87 (86%)	57 (90%)	0.47[b]	114 (88%)	30 (86%)	0.77[b]
	Present	14 (14%)	6 (10%)		15 (12%)	5 (14%)	
Invasion of the optic nerve	None	81 (80%)	52 (83%)	0.93[a]	107 (83%)	26 (74%)	0.34[a]
	Optic nerve head	18 (18%)	10 (16%)		20 (16%)	8 (23%)	
	Optic nerve	2 (2%)	1 (2%)		2 (2%)	1 (3%)	
Necrosis	No	81 (86%)	55 (89%)	0.81[b]	106 (86%)	30 (91%)	0.57[b]
	Present	13 (14%)	7 (11%)		17 (14%)	3 (9%)	
Marked pleomorphism	No	98 (97%)	49 (78%)	**<0.001**[b]	112 (87%)	35 (100%)	**0.025**[b]
	Present	3 (3%)	12 (22%)		17 (13%)	0 (0%)	
Tumor-infiltrating lymphocytes	No	92 (91%)	53 (84%)	0.21[b]	114 (88%)	31 (89%)	>0.99[b]
	Present	9 (9%)	10 (16%)		15 (12%)	4 (11%)	
Multinucleated giant cells	No	85 (84%)	40 (63%)	**0.004**[b]	91 (71%)	34 (97%)	**<0.001**[b]
	Present	16 (16%)	23 (37%)		38 (29%)	1 (3%)	
Nuclear pseudoinclusions	No	-	-	-	75 (58%)	26 (74%)	0.12[b]
	Present	-	-		54 (42%)	9 (26%)	
Intranuclear grooves	No	75 (74%)	54 (86%)	0.12[b]	-	-	-
	Present	26 (26%)	9 (14%)		-	-	-
Nucleoli presence	Low	5 (5%)	1 (2%)	0.41[b]	4 (3%)	2 (6%)	0.61[b]
	High	96 (95%)	62 (98%)		125 (97%)	33 (94%)	
Nucleoli size	No or micronucleoli	73 (72%)	26 (41%)	**<0.001**[b]	4 (3%)	31 (89%)	**<0.001**[b]
	Macronucleoli	28 (28%)	37 (59%)		64 (50%)	4 (11%)	
Nucleoli number	Low (0 or 1)	86 (85%)	37 (59%)	**<0.001**[b]	90 (70%)	33 (94%)	**<0.002**[b]
	High (2 or more)	15 (15%)	26 (41%)		39 (30%)	2 (6%)	
Pigmentation	Amelanotic	13 (13%)	1 (2%)	**<0.001**[a]	10 (8%)	4(11%)	0.33[a]
	Lightly pigmented	62 (61%)	25 (40%)		66 (51%)	21 (60%)	
	Heavily pigmented	26 (26%)	37 (59%)		53 (41%)	10 (29%)	
Hemorrhage	No	82 (81%)	48 (76%)	0.55[b]	99 (77%)	31 (89%)	0.16[b]
	Present	19 (19%)	15 (24%)		30 (23%)	4 (11%)	

[a]Chi-square test; [b]Fisher's exact test. Statistically significant results ($p<0.05$) are in bold text.

and DFS rates, Kaplan–Meier curves and the log-rank test were used; all analyses were carried out using the survival package for R. In order to determine the correlations between the presence of NPIs and NGs and continuous variables, the Wilcoxon two-sample test was used. The correlations between the presence of NPIs and NGs and binary variables were determined using Fisher's exact test while the correlations with other categorical variables were determined using the chi-square test. A *p*-value below 0.05 was considered significant for all comparisons.

Results

The study group comprised 164 patients, with 84 women and 80 men, aged between 18 and 86 years (on average 59.7 years). The observation time was on average 374.7 weeks (range=36-893 weeks). Other clinical and histopathological data of the tumors examined are presented in Tables I and II.

NPIs and NGs in UM. NPIs were noted in UM cells in 38% (63/164) and NGs in 21% (35/164) of all cases (Figure 1).

NPIs and NG – correlations with clinical parameters. Statistically significant correlations with clinical parameters were found only for NGs. Patients with NGs were significantly younger ($p=0.02$). The statistical analysis also showed a trend for the presence of NGs being inversely related to the degree of pigmentation ($p=0.057$).

There was no correlation between the presence of NGs and other evaluated clinical parameters, such as sex, involved eye,

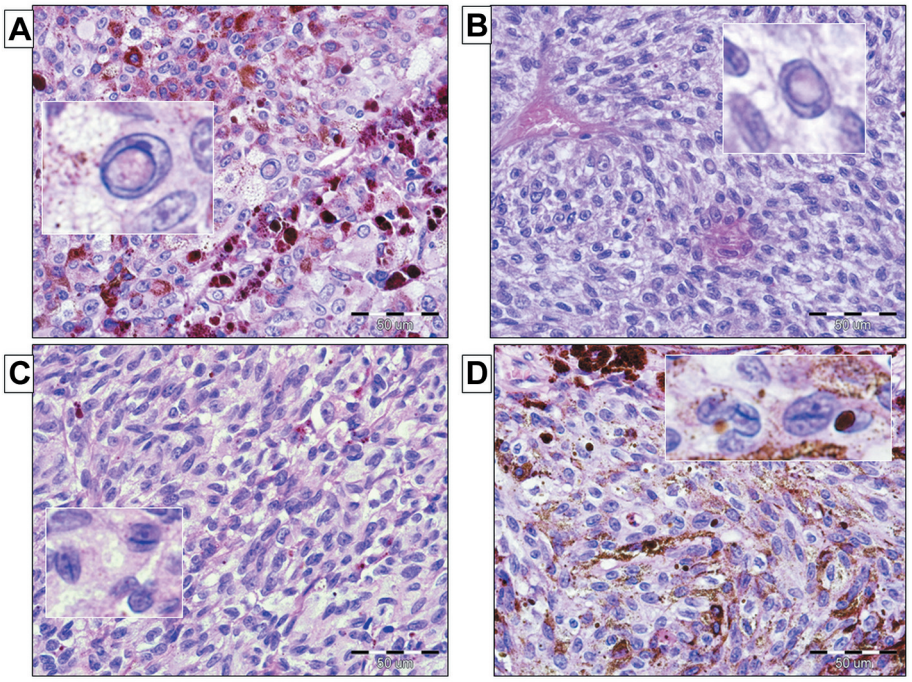

Figure 1. *Cytomorphology of nuclear pseudoinclusions and nuclear grooves in uveal melanoma cells. A, B: Prominent nuclear pseudoinclusions (inset) in uveal melanoma cells. C, D: Nuclear grooves (inset) in uveal melanoma cells. Hematoxylin and eosin staining, main image: 400×, inset: 600×.*

largest basal tumor diameter and thickness (by AJCC and COMS), stage, primary tumor shape, ciliary body involvement nor concomitant retinal detachment or glaucoma. There were no statistically significant correlations between the presence of NPIs and any analyzed clinical features.

Table I presents the relationship between NPIs and NGs in UM cells and clinical parameters.

NPIs and NGs – correlations with histological parameters. The presence of NPIs was significantly positively correlated with the mixed and epithelioid type of UM (p=0.0028), marked pleomorphism (p=0.00013), the presence of multinucleated giant cells (p=0.0043) and the presence of macronucleoli and multiple nucleoli (p<0.001 in both cases). Additionally, there was a positive correlation between the pigment content in tumor cells and the presence of NPIs (p=0.00003).

The presence of NGs was significantly inversely correlated with marked pleomorphism (p=0.025, lack of NGs in cases with marked histopathological pleomorphism), the presence of multinucleated giant cells (p=0.00054) and the presence of macronucleoli and multiple nucleoli (p<0.001 in both cases).

Table II presents relations between NPIs and NGs in UM cells and histological parameters.

The association of the presence NPIs and NGs with long-term survival. In the Kaplan–Meier analysis, the presence of NPIs was associated with significantly reduced overall survival (p<0.0001), however, this feature had no significant correlation with disease-free survival (p=0.1) (Figure 2).

The Kaplan–Meier analysis demonstrated that the presence of NGs was associated with significantly better overall (p=0.0016) and disease-free (p=0.029) survival (Figure 3).

Discussion

Histopathological diagnostics of tumors is based on the evaluation of differences between the structure of a normal tissue from which the tumor developed and the tumor tissue, as well as evaluations of the detailed morphology of analyzed cells. Changes in cell appearance which might be indicative of its potential for cancerous transformation are comprised of the morphological parameters of the cellular nuclei: Changes in their morphology may be a sign of a commencing or ongoing malignant transformation. These may consist of the formation of long inward folds of the nuclear envelope (NGs) and spherical invaginations of cytoplasm projecting partially into the nucleus (NPIs). The presence of NPIs and NGs is a typical feature of some tumors and their presence is important for pathologists in

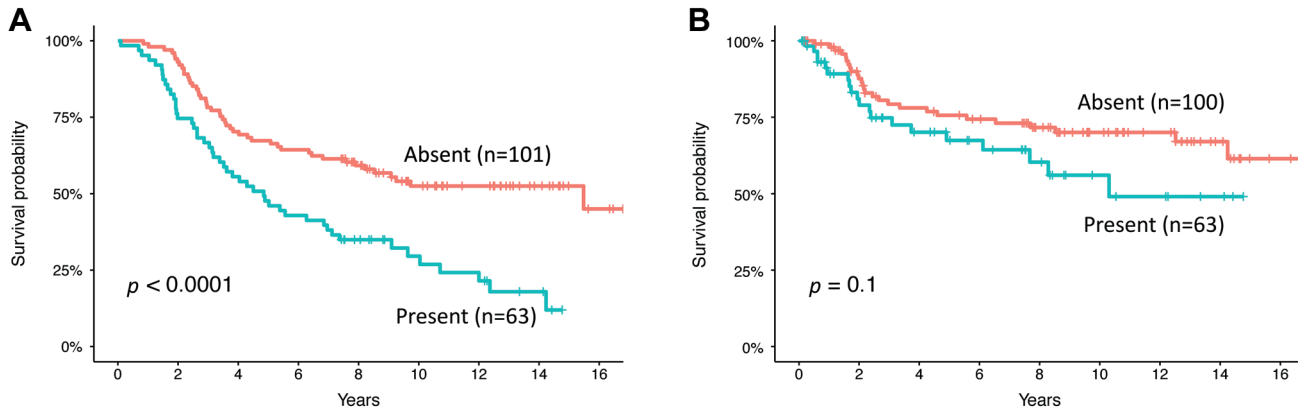

Figure 2. *Kaplan–Meier analysis of the prognostic impact of nuclear pseudoinclusions (NPIs) in patients with uveal melanoma. The presence of NPIs in melanoma cells was significantly associated with shorter overall survival (A) but not with disease-free survival (B).*

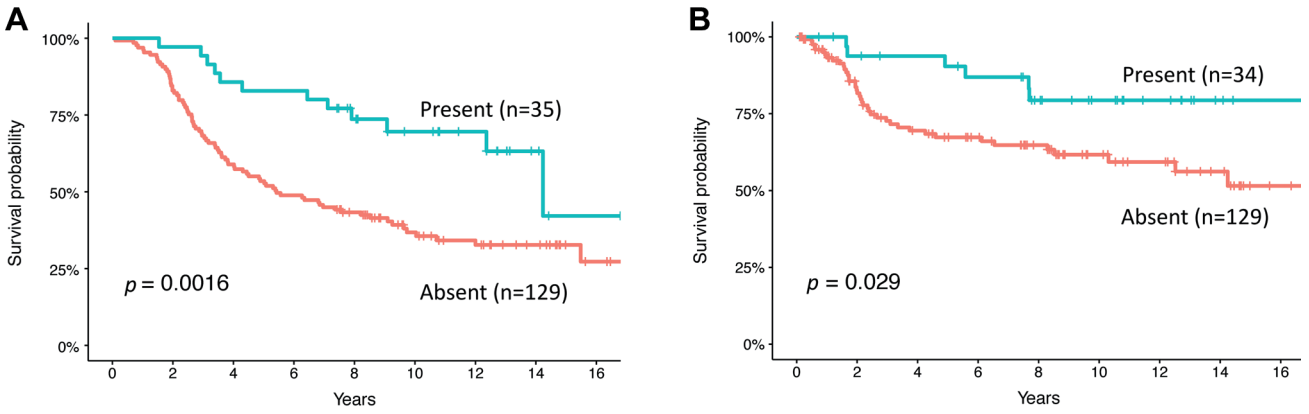

Figure 3. *Kaplan–Meier analysis of the prognostic impact of intranuclear grooves (NGs) in patients with uveal melanoma. The presence of NGs in melanoma cells was significantly associated with shorter overall survival (A) and disease-free survival (B).*

proper diagnosis, for example in papillary thyroid carcinoma and meningioma (19-22, 37-39).

In our study we decided to evaluate whether the presence of NPIs and NGs is indicative of a connection with clinical and histological features and long-term survival in patients diagnosed with a UM.

We observed the presence of NPIs in 38% (63/164) and NGs in 21% (35/164) of all UM cases, and the tumors involved mainly the ciliary body and choroid, occasionally also involving the iris. An interesting issue is that among our patients, both of these features occurred at the same time in only 9/164 cases, which makes up 5%. This might suggest that the presence of NPIs or NGs may have unrelated prognostic significance in UM.

There are only a few articles reporting the presence of NPIs and NGs in UM (31, 40-42). The first report concerning the presence of NPIs in UMs was presented in 1977 by Lommatzsch *et al.*, whilst detailed characteristics of these lesions in iris melanoma was made in 1980 by Sunba *et al.* (31, 40). They noted that similar structures were also observed in the melanoma of the ciliary body and the choroid, but these results were not published. In our study, the presence of NPIs was significantly positively correlated with the epithelioid type, marked pleomorphism, the presence of multinucleated giant cells and the presence of macronucleoli and multiple nucleoli, as well as histopathological properties indicative of a poorer prognosis. Additionally, there was also a positive correlation between the pigment content in tumor cells and the presence of NPIs. Importantly, patients with UM in whom NPIs are present in the tumor cells had significantly reduced overall survival ($p<0.0001$). Our observations in the case of NPIs differ from

those published by Sunba *et al*., who suggested that the presence of NPIs in UM may point to a more positive course of the disease. In the above report, NPIs were present in 60 (32%) cases out of 190 iris melanomas, of which 57 were of the spindle-cell type (31). It must be observed, however, that in their study, only iris UM cases were evaluated, and the majority of them were of the spindle-cell type, which is *a priori* associated with a better prognosis. Our analyses concerned mostly tumors involving the ciliary body and the choroid, which have poorer prognoses (6, 31). Additionally, we observed that NPIs were significantly more often noted when epithelioid cells were present, *i.e*. in the mixed type and in the epithelioid-cell type UM (p=0.0028). A similar observation concerning adverse prognosis in the case of the presence of NPIs in the cells of renal cell carcinoma was made by Ju-Han *et al*. They noted that the presence of a high number of NPIs may be an independent indicator of poor prognosis in renal cell carcinoma (38).

Another features of the nuclei of cancer cells which may play a role in the diagnostic process is the presence of NGs. In our study NGs were found in 21% (35/164) of all UM cases. Davila *et al*. in 1998 (41) and Kashyap *et al*. in 2002 (42) found the presence of NGs in individual cases of choroidal melanoma (2/9 and 1/1 respectively). They observed NGs only in spindle-shaped tumor cells. Likewise, in our cases, NGs had a tendencies for a more frequent occurrence in the spindle-cell type, which had a better prognosis.

Analysis of the presence of NGs with the clinical features in UM did not present any significant correlation, except that patients with NGs were younger (p=0.02) and in situations when the tumor was located anteriorly from the eye equator, *i.e*. with the involvement of the ciliary body (p=0.038). Histopathological features are a different issue. The presence of NGs was significantly inversely correlated with marked pleomorphism (we did not observe a single NGs-positive case with marked pleomorphism), the presence of multinucleated giant cells and the presence of macronucleoli and multiple nucleoli. Additionally, when NGs were present in the tumor cells in UM cases, the Kaplan–Meier analysis demonstrated significantly better overall (p<0.01) and disease-free (p<0.05) survival. These results would suggest that the presence of NGs in UM cells speaks in favor of a better prognosis, as opposed to the presence of NPIs, which is associated with the presence of histopathological properties associated with a poorer clinical outcome.

Several reports have indicated that in UM, the risk of metastasis increases with increased tumor pigmentation (5, 43). In our analyzed cases, we observed that the frequency of cases with NPIs significantly increased with the increase of melanin in the tumor cells (p=0.00003). However, the situation is completely different in the case of NGs because the statistical analysis showed a converse trend (p=0.057) indicating the presence of NGs was more frequent in amelanotic lesions.

In 2014, Fisher presented interesting theories describing the formation of NPIs and NGs (44). He believes that lesions like NPIs and NGs may have some genetic origin (develop in a near-diploid, genetically stable background) and that their formation is associated with tyrosine receptor kinase genes and probably with B-Raf proto-oncogene, serine/threonine kinase (*BRAF)* gene mutations, as is the case in papillary thyroid carcinoma, and also in melanoma and Langerhans cell histiocytosis. Additionally, somatic missense mutations (402C»G) at C134W (amino acid position 134) in the oncogene Forkhead box protein L2 (*FOXL2*) may be related to NGs in adult granulosa cell tumors of the ovary (44). What remains especially interesting is the fact that the *FOXL2* gene is located on chromosome 3, disorders of which (monosomy) are found in more than 50% of UMs (44-46).

Analysis of the connection between the presence of the *FOXL2* mutation and the mutations of other genes encoding transcription factors in UM requires further research.

It is beyond all doubt that the study of the significance of the presence of NPIs and NGs in UM requires further research carried out on a larger patient group, including of iris melanoma. Certainly, the relationship between NPIs or NGs and such factors as *BAP1* status, copy number changes in chromosomes 3 and 8q and sequencing of *BAP1* and *EIF1AX and* should be analyzed and also the statistical analysis should be extended with multivariate statistical models with reference to molecular analyses, clinical and histopathological features (47). Further studies are also necessary to find the causes of NPI and NG formation and the mechanism involved because the results of such research might cast some light on the role performed by these structures.

It would be also interesting to analyze melanocytic nevi with respect to the presence of NPIs, NGs and such factors as *BAP1* status, copy-number changes in chromosomes and different molecular disturbances, as was done in the case of benign pigmented skin lesions (48, 49). However, this task will be by no means simple as the study material may only be obtained either as a result of eyeball enucleation, resection of tumor within the iris, ciliary body or anterior choroid and fine-needle biopsy – none of which are standard procedures in uveal melanocytic nevi.

Conclusion

To our knowledge, our study is the first to present such an extensive analysis of the presence of NPIs and NGs in UM in relation to the clinical and histological properties of this tumor and its prognosis.

We have confirmed the presence of NPIs and NGs in UM. The presence of NPIs appears to be related to the

histopathological features of UM which are associated with poorer prognosis, which translates into significantly reduced overall survival. In contrast, NGs co-exist with cell morphological features which denote better prognosis and their presence also correlated with significantly better overall and disease-free survival. Comprehensive and large-scale studies should follow this preliminary report to evaluate the prognostic status of NPIs and NGs in UM cases and the nature and origin of these nuclear features.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

A.M.: Conceptualization, methodology, investigation, resources, data curation, writing - original draft; P.D.: conceptualization, methodology, investigation, resources, data curation, writing - review & editing, visualization, project administration, funding acquisition; P.B.: software, formal analysis, visualization, funding acquisition; J.O.H.: investigation, resources; E.M.: methodology, resources; writing - review & editing, funding acquisition; B.R.D.: investigation, resources, supervision; writing - review & editing; funding acquisition. All Authors reviewed the article and approved the submitted version.

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References

1 Kaliki S and Shields CL: Uveal melanoma: Relatively rare but deadly cancer. Eye *31*: 241-257, 2017. PMID: 27911450. DOI: 10.1038/eye.2016.275

2 Singh AD, Turell ME and Topham AK: Uveal melanoma: Trends in incidence, treatment, and survival. Ophthalmology *118*: 1881-1885, 2011. PMID: 21704381. DOI: 10.1016/j.ophtha.2011.01.040

3 Virgili G, Gatta G, Ciccolallo L, Capocaccia R, Biggeri A, Crocetti E, Lutz JM and Paci E: Incidence of uveal melanoma in Europe. Arch Ophthalmol *114(12)*: 2309-15, 2007. PMID: 17498805. DOI: 10.1016/j.ophtha.2007.01.032

4 Kivelä T: The epidemiological challenge of the most frequent eye cancer: Retinoblastoma, an issue of birth and death. Br J Ophthalmol *93*: 1129-1131, 2009. PMID: 19704035. DOI: 10.1136/bjo.2008.150292

5 Shields CL, Kaliki S, Cohen MN, Shields PW, Furuta M and Shields JA: Prognosis of uveal melanoma based on race in 8100 patients: The 2015 Doyne Lecture. Eye *29*: 1027-1035, 2015. PMID: 26248525. DOI: 10.1038/eye.2015.51

6 Jager MJ, Shields CL, Cebulla CM, Abdel- Rahman MH, Grossniklaus HE, Stern MH, Carvajal RD, Belfort RN, Jia R, Shields JA and Damato BE: Uveal melanoma. Nat Rev Dis Primers *6(1)*: 24, 2020. PMID: 32273508. DOI: 10.1038/s41572-020-0158-0

7 Rodríguez A, Dueñas-Gonzalez A and Delgado-Pelayo S: Clinical presentation and management of uveal melanoma (Review). Mol Clin Oncol *27*: 675-677, 2016. PMID: 28101347. DOI: 10.3892/mco.2016.1037

8 Shields CL, Furuta M, Thangappan A, Nagori S, Mashayekhi A, Lally DR, Kelly CC, Rudich DS, Nagori AV, Wakade OA, Mehta S, Forte L, Long A, Dellacava EF, Kaplan B and Shields JA: Metastasis of uveal melanoma millimeter-by-millimeter in 8033 consecutive eyes. Arch Ophthalmol *127*: 989-998, 2009. PMID: 1966733. DOI: 10.1001/archophthalmol.2009.208

9 Berus T, Halon A, Markiewicz A, Orlowska-Heitzman J, Romanowska-Dixon B and Donizy P: Clinical, histopathological and cytogenetic prognosticators in uveal melanoma – A comprehensive review. Anticancer Res *37(12)*: 6541-6549, 2017. PMID: 29187428. DOI: 10.21873/anticanres.12110

10 McLean IW, Foster WD, Zimmerman LE and Gamel JW: Modifications of Callender's classification of uveal melanoma at the Armed Forces Institute of Pathology. Am J Ophthalmol *96*: 502-509, 1983. PMID: 6624832. DOI: 10.1016/s0002-9394(14)77914-0

11 Folberg R, Rummelt V, Parys-Van Ginderdeuren R, Hwang T, Woolson RF, Pe'er J and Gruman LM: The prognostic value of tumor blood vessel morphology in primary uveal melanoma. Ophthalmology *100*: 1389-1398, 1993. PMID: 8371929. DOI: 10.1016/s0161-6420(93)31470-3

12 Bronkhorst IH, Vu TH, Jordanova ES, Luyten GP, Burg SH and Jager MJ: Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Invest Ophthalmol Vis Sci *53*: 5370-5378, 2012. PMID: 22743317. DOI: 10.1167/iovs.11-9280

13 Rantala ES, Hernberg M and Kivelä TT: Overall survival after treatment for metastatic uveal melanoma: A systematic review and meta-analysis. Melanoma Res *29(6)*: 561-568, 2019. PMID: 30664106. DOI: 10.1097/CMR.0000000000000575

14 Harbour JW, Onken MD, Roberson EDO, Duan S, Cao L, Worley LA, Council ML, Matatall KA and Helms C: Frequent mutation of BAP1 in metastasizing uveal melanomas. Science *330*: 1410-1413, 2010. PMID: 21051595. DOI: 10.1126/science.1194472

15 Sipos E, Hegyi K, Treszl A, Steiber Z, Mehes G, Dobos N, Fodor K, Olah G, Szekvolgyi L, Schally AV and Halmes G: Concurrence of chromosome 3 and 4 aberrations in human uveal melanoma. Oncol Rep *37(4)*: 1927-1934, 2017. PMID: 28350068. DOI: 10.3892/or.2017.5496

16 Shields JA and Shields CL: Management of posterior uveal melanoma: past, present, and future: The 2014 Charles L. Schepens Lecture. Ophthalmology *122*: 414-428, 2015. PMID: 25439609. DOI: 10.1016/j.ophtha.2014.08.046

17 Arora SK and Dey P: Intranuclear peudoinclusions: Morphology, pathogenesis and significance. Diagn Cytopathol *40*: 741-744, 2012. PMID: 21548120. DOI:10.1002/dc.21714

18 Ip YT, Dias Filho MA and Chan JKC: Nuclear inclusions and pseudoinclusions: Friends or foes of the surgical pathologist? Int

J Surg Pathol *18*: 465-481, 2010. PMID: 21081532. DOI: 10.1177/1066896910385342

19 Petrilli G, Fisogni S, Rosai J, Festa S, Torri L, Rodeschi A, Licini S and Facchetti F: Nuclear bubbles (nuclear pseudo-pseudoinclusions): A pitfall in the interpretation of microscopic sections from the thyroid and other human organs. Am J Surg Pathol *41*: 140-141, 2017. PMID: 27792063. DOI: 10.1097/PAS.0000000000000763

20 Das DK: Intranuclear cytoplasmic inclusions in fine-needle aspiration smears of papillary thyroid carcinoma: a study of its morphological forms, association with nuclear grooves, and mode of formation. Diagn Cytopathol *32(5)*: 264-268, 2005. PMID: 15830362. DOI: 10.1002/dc.20233

21 Das DK and Sharma PN: Intranuclear cytoplasmic inclusions and nuclear grooves in fine needle aspiration smears of papillary thyroid carcinoma and its variants: advantage of the count under an oil-immersion objective over a high-power objective. Anal Quant Cytol Histol *27(2)*: 83-94, 2005. PMID: 15913201. DOI 10.1159/000326381

22 Yoshida T, Hirato J, Sasaki A, Yokoo H, Nakazato Y and Kurachi H: Intranuclear inclusions of meningioma associated with abnormal cytoskeletal protein expression. Brain Tumor Pathol *16*: 86-91, 1999. PMID: 10746966. DOI: 10.1007/BF02478908

23 Yang SW, Yang KM, Kang HY and Kim T: Intranuclear cytoplasmic pseudoinclusions in pituitary adenomas. Yonsei Med J *44*: 816-820, 2003. PMID: 14584097. DOI: 10.3349/ymj.2003.44.5.816

24 Kobayashi S, Saio M, Fukuda T, Kimura K, Hirato J and Oyama T: Image analysis of the nuclear characteristics of emerin protein and the correlation with nuclear grooves and intranuclear cytoplasmic inclusions in lung adenocarcinoma. Oncology Rep *41*: 133-142, 2019. PMID: 30542735. DOI: 10.3892/Or.2018.6848

25 Gould E, Watzak L, Chamizo W and Albores-Saavedra J: Nuclear grooves in cytologic preparations. A study of the utility of this feature in the diagnosis of papillary carcinoma. Acta Cytol *33(1)*: 16-20, 1998. PMID: 2536993.

26 Novak JA and Masood S: Nuclear grooves in fine-needle aspiration biopsies of breast lesions: Do they have any significance? Diagn Cytopathol *18(5)*: 333-337, 1998. PMID: 9582567. DOI: 10.1002/(sici)1097-0339(199805)18:5<333::aid-dc5>3.0.co;2-b

27 Kumar PV: Nuclear grooves in ependymoma. Cytologic study of 21 cases. Acta Cytol *41(6)*: 1726-1731, 1997. PMID: 9390132. DOI: 10.1159/000333176

28 Tahlan A and Dey P: Nuclear grooves. How specific are they? Acta Cytol *45(1)*: 48-50, 2001. PMID: 11213503. DOI: 10.1159/000327186

29 Donizy P, Kaczorowski M, Biecek P, Halon A and Matkowski R: Nuclear pseudoinclusions in melanoma cells: prognostic fact or artifact? The possible role of Golgi phosphoprotein 3 overexpression in nuclear pseudoinclusions generation. Pathol Int *68*: 117-122, 2018. PMID: 29377340. DOI:10.1111/pin.12629

30 Kashyap S, Sen S, Sharma MC and Sethi A: Diagnostic intraocular aspiration cytology of choroidal melanoma. Diagn Cytopathol *26(6)*: 389-391, 2002. PMID: 12112831. DOI: 10.1002/dc.10118

31 Sunba MSN, Rahi AHS and Morgan G: Tumours of the anterior uvea. II. Intranuclear cytoplasmic inclusions in malignant melanoma of the iris. Br J Ophthalmol *64*: 453-456, 1980. PMID: 6248099. DOI: 10.1136/bjo.64.6.453

32 Edge SD, Byrd DR and Compton CC: Malignant melanoma of the uvea. AJCC Cancer Staging Manual. Seventh Edition. New York, NY: Springer pp. 547-560, 2010.

33 Grossniklaus HE, Finger PT, Harbour JW and Kivëla T: Protocol for the Examination of Specimens From Patients With Uveal Melanoma, 2017. Available at: https://documents.cap.org/protocols/cp-uveal-melanoma-17protocol-4000.pdf

34 Margo CE: The Collaborative Ocular Melanoma Study: An overview. Cancer Control *11(5)*: 304-309, 2004. PMID: 15377989. DOI: 10.1177/107327480401100504

35 R Core Team: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2019. Available at: https://www.r-project.org/

36 Kassambara, A.; Kosinski, M.; Biecek, P.; Fabian, S. Survminer: Drawing Survival Curves using "ggplot2". R package version 0.4.6. 2019. Available at: https://cran.r-project.org/package=survminer

37 Shurbaji MS, Gupta PK and Frost JK: Nuclear grooves: A useful criterion in the cytopathologic diagnosis of papillary thyroid carcinoma. Diagn Cytopathol *4(2)*: 91-94, 1998. PMID: 2468463. DOI: 10.1002/dc.2840040202

38 Lee JH, Han EM, Lin ZH, Wu ZS, Lee ES and Kim YS: Clinicopathologic significance of nuclear grooves and inclusions in renal cell carcinoma. Image database construction and quantitative scoring. Arch Pathol Lab Med *132*: 940-946, 2008. PMID: 18517276. DOI: 10.1043/1543-2165(2008)132[940:CSONGA]2.0.CO;2

39 Olins AL and Olins DE: Cytoskeletal influences on nuclear shape in granulocytic HL-60 cells. BMC Cell Biol *5*: 30, 2004. PMID: 15317658. DOI: 10.1186/1471-2121-5-30

40 Lommatzsch P and Klug H: Ein beitrag zur ultrastrukur des irismelanoms. Albrecht von Graffes Arch Klin Ophthalmol *203*: 101-117, 1977.

41 Dávila RM and Miranda MC and Smith ME: Role of cytopathology in the diagnosis of ocular malignancies. Acta Cytol *42(2)*: 362-366, 1998. PMID: 9568136. DOI: 10.1159/000331616

42 Kashyap S, Sen S, Sharma MC and Sethi A: Diagnostic intraocular aspiration cytology of choroidal melanoma. Diagn Cytopathol *26(6)*: 389-391, 2002. PMID: 12112831. DOI: 10.1002/dc.10118

43 Janjetovic Z, Brozyna AA, Tuckey RC, Kim TK, Nguyen MN, Jozwicki W, Pfeffer SR, Pfeffer LM and Slominski AT: High basal NF-kB activity in nonpigmented melanoma cells is associated with an enhanced sensitivity to vitamin D3 derivatives. Br J Cancer *105*: 1874-1884, 2011. PMID: 22095230. DOI: 10.1038/bjc.2011.458

44 Fischer AH: The diagnostic pathology of the nuclear envelope in human cancers. Adv Exp Med Biol *773*: 49-75, 2014. PMID: 24563343. DOI: 10.1007/978-1-4899-8032-8_3

45 Leung DT, Fuller PJ and Chu S: Impact of FOXL2 mutations on signaling in ovarian granulosa cell tumors. Int J Biochem Cell B *72*: 51-54, 2016. PMID: 26791928. DOI: 10.1016/j.biocel.2016.01.003

46 Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jöckel KH and Becher R: Prognostic implications of monosomy 3 in uveal melanoma. Lancet *347*: 1222-1225, 1996. PMID: 8622452. DOI: 10.1016/s0140-6736(96)90736-9

47 Robertson AG, Shih J, Yau Ch, Gibb EA, Oba J, Mungall KL, Hess J.M, Uzunangelov V, Walter V, Danilova L, Lichtenberg TM, Kucherlapati M, Kimes PK, Tang M, Penson A, Babur O,

Akbani R, Bristow CA, Hoadley KA, Iype L, Chang MT, TCGA Research Network; Cherniack AD, Benz C, Mills GB, Verhaak RGW, Griewank KG, Felau I, Zenklusen JC, Gershenwald JE, Schoenfield L, Lazar AJ, Abdel-Rahman MH, Roman-Roman S, Stern MH, Cebulla CM, Williams MD, Jager MJ, Coupland SE, Esmaeli B, Kandoth C and Woodman SE: Cancer integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell *32*: 204-220, 2017. PMID: 28810145. DOI: 10.1016/j.ccell.2017.07.003

48 Rose DSC: Nuclear pseudoinclusions in melanocytic naevi and melanomas. J Clin Pathol *48*: 676-677, 1995. PMID: 7560180. DOI: 10.1136/jcp.48.7.676

49 Valdebran M, Elbendary A, Chaitanya Arudra SK, Torres KM, Elattar I and Elston DM: Nuclear and cytoplasmic features in the diagnosis of banal nevi, Spitz nevi, and melanoma. J Am Acad Dermatol *75*: 1032-1037, 2016. PMID: 27542585. DOI: 10.1016/j.jaad.2016.06.051

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