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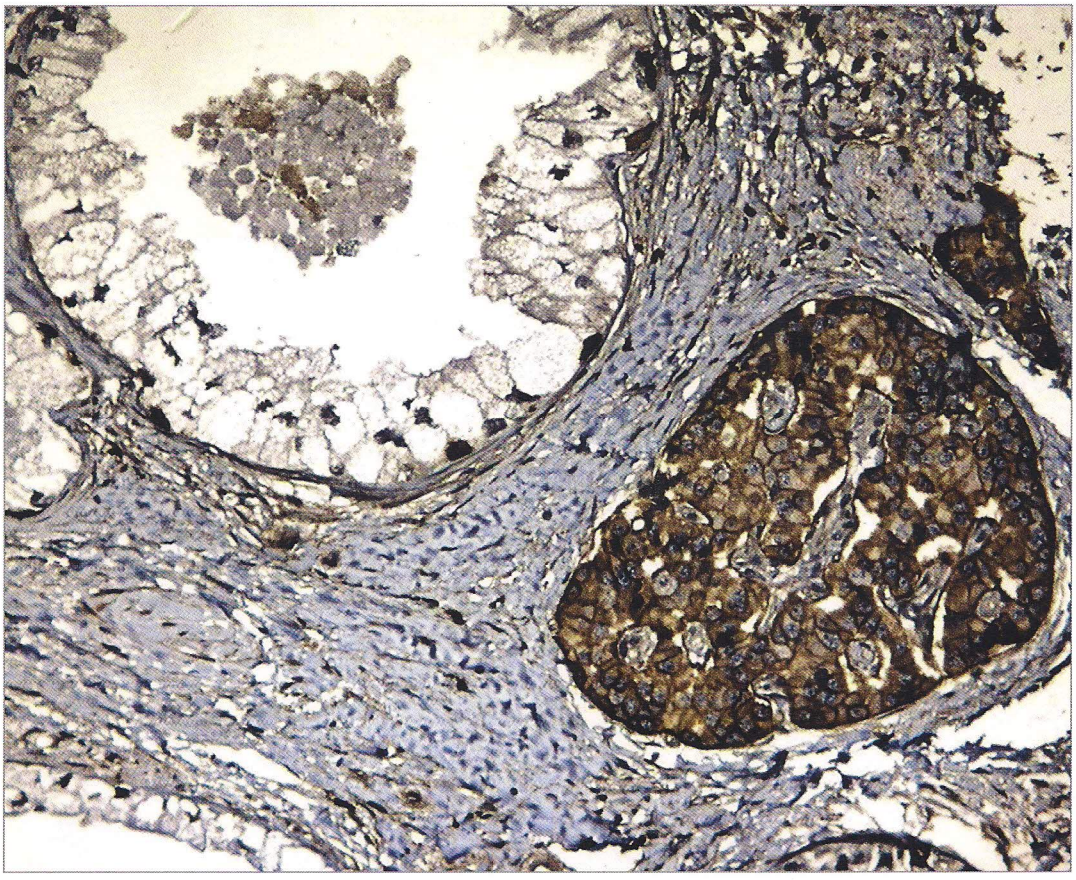

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АРХИВ ПАТОЛОГИИ

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Адрес редакции:
125284 Москва, ул. Поликарпова, д. 12.
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E-mail: arh.pat@gmail.com
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Immunohistochemical characteristics of uveal melanoma assording to the age at diagnosis, histological type and extension of the tumor

N.V. DANILOVA, S.YU. DAVYDOVA

[1]Faculty of Fundamental medicine of M.V. Lomonosov Moscow State University (Moscow, 119192, Russian Federation); [2]Pathology department of Russian Postgraduate Medical Academy (Moscow, 123995, Russian Federation)

Иммуногистохимические особенности увеальной меланомы в зависимости от гистологического типа, степени инвазии и возраста пациента

Н.В. ДАНИЛОВА[1, 2], С.Ю. ДАВЫДОВА[1]

[1]Факультет фундаментальной медицины МГУ им. М.В. Ломоносова, 119192, Москва; [2]ГБОУ «ДПО Российская медицинская академия последипломного образования», 123995, Москва

The purpose of this study was to investigate the relationship between MMP9 expression and tumour invasion in different structures of the eye. We also examined whether there was any correlation between the growth factors (TGFb and EGF), oncosuppressor proteins (p16 and p53) and Ki-67, and the tumour histological subtypes, atypia level and age at diagnosis. Tumour specimens were obtained from 42 primary uveal melanomas immediately after enucleation at The Helmholtz Moscow Research Institute of Eye Diseases. The patients were not treated with radio- or thermotherapy. During our systematic study, we exclusively employed 10%-formalin fixed, paraffin-wax-embedded tissue sections of UM for histological diagnosis and immunohistochemistry. According to our data the hyperexpression of MMP9 and EGFR correlates with a high proportion of spindle cells in a tumour (Kruskal—Wallis test p=0,1 for each). Moreover, we have demonstrated the association between the level of EGFR, TGFb and MMP9 expression and the initial invasion stage (Spearman's test p=0,1). In addition, we have revealed the significant correlation between TGFb hyperexpression and atypia level (Spearman's test p=0,059). Our data reflect that the diagnoses at an advanced age correlate with hyperexpression of p16 (Kruskal—Wallis test p=0,068). An interesting result is that p16 level reduced in inverse proportion to that of TGFb. On the basis of our data and previous studies, we reached the conclusion that after the lapse of time the level of p16 rises significantly in order to inhibit proliferating activity of melanocytes in the normally functioning pigmented layer. However, although the probability of UM diagnoses in elderly is increasing, we have no reliable data for the relationship with high atypia levels.

Key words: uveal melanoma, immunohistochemistry, TGFb, paxient age.

Увеальная меланома (УМ) — одна из наиболее часто встречающихся внутриглазных злокачественных опухолей. Несмотря на значительный прогресс в области лучевой и химиотерапии УМ, примерно у 40—50% пациентов с большим первичным опухолевым узлом развиваются отдаленные метастазы, а смерть от опухолевой прогрессии наступает в первые годы после операции. В связи с отсутствием лимфатического дренирования увеального тракта, УМ метастазирует только гематогенным путем преимущественно в печень (95%), легкие (24%), кости (16%) и кожу (11%). В последнее 10-летие активно обсуждаются увеличение доли молодых пациентов (до 20—35 лет) с диагнозом УМ на фоне общего снижения возрастной планки при постановке диагноза (от 55—60 лет) и влияние различных морфологических факторов на прогноз в этой группе. При этом активно изучается экспрессия различных иммуногистохимических маркеров (Ki-67, p53 и p16, TGFb и EGFR, и MMP9) и их роль в патогенезе меланомы сосудистого тракта. **Цель исследования** — определить иммуногистохимические особенности УМ в зависимости от гистологического типа, степени инвазии и возраста пациентов. **Материал и методы.** Исследование проведено на архивном материале от 42 пациентов МНИИ глазных болезней им. Г. Гельмгольца за период с января 2011 по июль 2012 г. Из них 24 мужчины и 18 женщин в возрасте от 15 до 74 лет (основной возраст постановки диагноза 58 лет, средний возраст 44,5 года). Все пациенты были разделены на 3 возрастные группы: до 35 лет (7 пациентов), от 35 до 55 лет (13 пациентов) и от 55 лет (22 пациента). Образцы опухоли, взятые из боковой колодки глаза после энуклеации, фиксировали в 10% забуференном формалине в течение 24—72 ч и заливали в парафиновые блоки. С каждого блока были изготовлены 4-μк срезы, которые монтировали на высокоадгезивные стекла. Иммуногистохимическое окрашивание и обработку материала проводили с использованием автостейнера Dako. Депарафинирование и регидратацию проводили с помощью системы En Visison Flex («Dako», Дания) при температуре 95—98 °С и pH 9,0 в течение 20 мин в модуле предобработки к автостейнеру (PT-module). В качестве хромогена использовали DAB. Анализ результатов проводился с использованием программы Stata 12 по методу Краскела—Уоллиса, который является многомерным обобщением ***U***-теста Манна—Уитни для ранговых данных. Для оценки уровня корреляции использовался тест Спирмена, который также является ранговым. Уровень значимости (p) принимался равным 0,1 для каждого из методов. **Результаты и обсуждение.** В результате исследования подтвердилось наличие достоверной корреляции между количеством митозов и высокой экспрессией TGFb (по тесту Спирмена p=0,059), при этом уровень экспрессии TGFb обратно пропорционален уровню p16. Генетические исследования зарубежных авторов выявили, что для пациентов с УМ характерно наличие мутаций в хромосоме 3p22, которая кодирует в том числе ген *TGFbR2*, который в свою очередь ведет к изменениям в SMAD-каскаде (а именно SMAD типов 2, 3 и 4), запускающих синтез целого семейства ингибиторов Cdk. На основании приведенных данных можно предположить, что полученные результаты свидетельствуют в пользу того, что патогенез УМ напрямую связан с мутациями в генах, кодирующих TGFb и его рецепторы, которые ведут к нарушению регуляции клеточного цикла через угнетение синтеза p16 и как следствие увеличению количества митозов. Нами установлено, что высокий

уровень экспрессии p16 ассоциирован с более поздним выявлением УМ (по тесту Краскела—Уоллиса p=0,068). На основании вышеизложенного можно сделать вывод, что в норме с возрастом количество p16 должно увеличиваться, угнетая тем самым пролиферацию меланоцитов пигментного эпителия сетчатки. Но с увеличением возраста пациента вероятность диагностировать УМ резко возрастает. Более того, по нашим данным не выявлено корреляции между уровнем p16 и степенью атипии в опухоли (по тесту Краскела—Уоллиса p=0,924). Для подтверждения гипотезы необходимо проведение дальнейших генетических исследований мутаций в гене *p16* с учетом возраста постановки диагноза. Относительно влияния различных маркеров на клеточный тип опухоли, нами выявлена тенденция к увеличению уровня EGFR с уменьшением доли эпителиоидных клеток в опухоли (по тесту Краскела—Уоллиса p=0,1). Помимо этого, высокая экспрессия MMP9 коррелирует с более благоприятным клеточным типом опухоли (по тесту Краскела—Уоллиса p=0,1), что противоречит данным литературы о наличии связи между высоким уровнем MMP9 и EGFR и большей доли эпителиоидных клеток в опухоли. Примечательно, что экспрессия маркеров MMP9, TGFb и EGFR связана с начальным этапом инвазии опухоли в структуры глаза. Наши данные противоречат существующей в литературе гипотезе об активации MMP9 на более поздних этапах диссеминирования УМ. **Заключение.** На основании полученных результатов можно предположить, что возможное использование таргетных препаратов рациональнее всего проводить при более доброкачественных формах УМ и начальных этапах инвазии как возможный способ избежать энуклеации пораженного глаза.

Ключевые слова: увеальная меланома, иммуногистохимия, TGFb, возраст пациента.

Uveal melanoma (UM) is the commonest type of primary intraocular malignant tumour. UM is the second most common form of melanoma and represents approximately 5—6% of all melanoma diagnoses [1]. Despite significant advances in the treatment of primary UM, approximately 40—50% of patients with large tumours develop metastases, with very poor survival rates after the discovery of metastatic disease, in spite of therapy [2]. Uveal melanoma cells are thought to metastasize primarily by hematogenous spread, due to a lack of lymphatic drainage in the uveal tract. Usually the metastases appear first in the liver in up to 95% of patients, in the lungs (24%), bone (16 %), and skin (11%) [2].

There is a tendency to believe that the mean age at diagnosis is the mid 60s [1], but the median age of UM presentation has fallen from 65 to 50—55 years due to greater proportion of younger patients (<20—35 years) through out the last decade [3]. The influence of different morphologic factors on this phenomenon is still under investigation by many authors. For instance, according to S. Kaliki et al., younger patients at the time of diagnosis of UM are associated with lower rates of metastasis compared to middle-aged and older adults [4]. Nevertheless, the remains a lack of knowledge about age impact on the immunohistochemical expression and morphologic parameters of UM.

An extensive body of literature exists on the expression of different immunohistochemical markers like Ki-67, p53, p16, TGFb, EGFR, MMP2 and MMP9 and their role in the pathogenesis of UM. The matrix metalloproteinase (MMP) family of enzymes is involved in the degradation of extracellular matrix components at the tumour—host tissue interface. This degradation is a key event in the tumour invasion [5]. As noted in recent reports, MMP2 and MMP9 were predominantly present in epitheloid melanomas or the epitheloid portion of mixed cell uveal melanomas, and high levels of expression were associated with a significantly higher incidence of metastatic disease [6—8]. On the other hand, little is known about the involvement of increased MMP-activity in specific invasions (such as optic nerve or anterior chamber angle, vitreous etc.) or in cell type of UM.

Ki-67 is a non-histone protein component of DNA polymerase, expressed in all non-G0 phases of the cell cycle (all proliferating cells) [9]. Because of that, Ki-67 monoclonal antibodies have become a robust test of proliferation activity in a wide variety of tumours. According to M. Mooy, there is a correlation between the Ki-67 index and the presence of the epitheloid cell type and apoptotic index [10]. However, although this marker is widely used as a significant predictive factor, there is no data about the relationship between the expression of Ki-67 and the atypia level or the patient's age.

The TP53 onco-suppressor gene encodes the cell-cycle regulator protein p53. The latter activates with DNA damage and leads to cell-cycle arrest or apoptosis in normal human cells. Recent studies have found that 50% of malignant tumours have mutations in gene TP53, which plays a crucial role in oncogenesis [11]. The study by J.S. Chana et al. demonstrates the absence of any correlation between the cell-type of UM and the level of marker expression. Moreover, according to their data, abnormalities in the p53-cascade are unlikely to have any effect on the progression of UM [12]. Nonetheless, according to S.E. Coupland et al., p53 was associated with unfavourable outcomes (death from metastases within the first 5 years after diagnosis of UM) and more aggressive tumour growth [13]. In other words, the potential role of p53 in tumorigenesis of UM remains unclear.

Protein p16 has been identified as an onco-suppressor in normal human cells. The inhibitory activity of p16 is restricted to the cyclin D-cdk4 and cyclin D-cdk6 kinases and results in cell cycle control at the G1-S restriction point. Recent studies found that the gene CDK^{N2A} (chromosome 9p21) encodes a putative cell cycle inhibitor, p16, and is frequently lost or rearranged in UM cell lines [14, 15]. The K. Lamperska et al. data show that increased levels of p16 correlate with the epitheloid type of UM, but do not have any association with tumour invasiveness [16]. In contrast, the results X. Wang et al. provide evidence against p16 having a significant role for p16 in intraocular melanomas [14]. Although p16 is identified as a potential player in the UM tumorigenic process, no definitive studies have shown this to be of either prognostic significance or to have any correlation with the patient's age.

TGFb is capable of suppressing the growth of normal human melanocytes, but melanoma cells lose this response both in skin skin and ocular tissue. It is noteworthy that TGFb is produced by the ciliary body and the retina within the eye and that binding to TGFbR1 results in the activation of biochemical pathways involving a series of SMAD proteins, which cause the up-regulation of a number of cdk-inhibitors including p16 [17].

Данилова Наталья Владимировна — к.м.н., асс. каф. физиологии и общей патологии; e-mail: natalyadanilova@gmail.com

The information about the primary antibodies

Protein	Antibody clone	Antibody dilution	Manufacturer
Ki-67	MIB1	RTU	Dako (Denmark)
p53	DO-7	1:30	Dako (Denmark)
p16	E6H4	1:25	Dako (Denmark)
EGFR	EGFR pharmDx Kit for Dako Autostainer		Dako (Denmark)
TGF-β	NCL-TGF-β	1:30	Leica Mycrosystems (Germany)
MMP-2	NCL-MMP2-507	1:100	Leica Mycrosystems (Germany)
MMP-9	NCL-MMP9-439	1:70	Leica Mycrosystems (Germany)

Some authors claim that the expression of endoglin (a transmembrane regulatory receptor on proliferating endothelium for TGFb) can be used as a specific marker for angiogenesis in uveal melanomas [18]. Unfortunately, the place and role of this growth factor in pathogenesis of UM is still unknown, as is the relationship to age at diagnosis, tumour cell-type and the atypia level.

The same situation exists with EGF (epidermal growth factor) that is normally synthesized in the liver and promotes the regenerative process. Even though the role of EGF in UM progression is still unknown, recent research into primary cutaneous melanoma demonstrates a significant correlation between EGFR alterations and histological subtypes, tumour thickness, ulceration and metastases formation [19]. Some authors have shown the association of EGFR-level and the metastatic potential of intraocular melanomas to the liver [20]. Contrary to the previous study, A. Kiss et al. [21] do not confirm the hypothesis of EGFR hyperexpression as a relevant prognostic factor in patients with UM. In fact, this little-investigated part of UM pathway needs further examination.

The purpose of this study was to investigate the relationship between MMP expression and tumour invasion in different structures of the eye. We also examined whether there was any correlation between the growth factors (TGFb and EGF), onco-suppressor proteins (p16 and p53) and Ki-67, and the tumour histological subtypes, atypia level and age at diagnosis.

Between January 2011 and July 2012, tumour specimens were obtained from 42 primary uveal melanomas immediately after enucleation at The Helmholtz Moscow Research Institute of Eye Diseases. The patients were not treated with radio- or thermotherapy. In 33 patients the melanoma was localized within the uvea. In 13 of these cases it was localized within the uvea and ciliary body, and 8 of them featured tumours with extrascleral growth pattern (including 7 cases, where tumours infiltrated the optic nerve). We included in our study UM with little pigmentation (28 tumours), some pigmentation (10 tumours) and no pigmentation (4 tumours). Histological diagnosis of UM assessed for cell type revealed: 21 cases of spindle, 7 cases of epitheloid and 15 of mixed type. Of the 42 patients studied, 18 were women and 27 were men. The age range was from 15 to 74 years with a mean age at diagnosis of 58 years. All patients were divided into three groups according to the age at diagnosis: young <35 years (7 cases), middle-aged adults 35—55 years (13 cases), and older adults >55 years (22 cases).

Levels of invasion were evaluated according to sclera infiltration or/and emissarium involvement: (0) — absence of growth, (1) — less than $^1/_3$ thickness or length involvement, (2) — about $^2/_3$ thickness or length involvement, (3) — infiltration of all sclera or/and an extra-/intrascleral growth pattern (including cases with more than $^2/_3$ length growth pattern in the emissary).

Tumour atypia was analysed by counting the number of cells with mitotic activity. The mitotic activity was determined by counting mitotic figures in 40 'high-power fields' (×400 m.p.) and divided into three groups: (1) — <10 mitotic figures, (2) — 10—40 mitotic figures, (3) — >40 mitotic figures.

During our systematic study, we exclusively employed 10%-formalin fixed, paraffin-wax-embedded tissue sections of 42 UM for histological diagnosis and immunohistochemistry. Each specimen was serially sectioned into 4-μm sections and mounted on poly-L-lysine-coated glass slides (Dako, Denmark) for immunohistochemical staining in Dako autostainer. After serial paraffin sections were made, they were deparaffinized and rehydrated with the EnVisison Flex system («Dako», Denmark) at 90—95 °C and pH 9.0 for 20 min in PT-module. After washing, antibody-treated (Table) sections were developed with 3-Diaminobenzidine and counterstained with Mayer's hematoxylin and coverslipping. Hematoxylin-eosin staining was performed in separate sections to determine the histological orientation.

The immunohistological specimens were evaluated by two independent observers. The stained sections were analysed for the expression of Ki67, p53, p16, TGFb, EGFR, MMP2, and MMP9. Unfortunately, MMP2 did not show any reaction, so it was excluded from the study.

The expression of MMP9, TGFb and EGFR in the specimen was classified by the semi-quantitative method (considering both the proportion of positive cells and intensity of staining in a ×40 objective in 5 fields): (–) negative; (+) weak; (++) moderate, or high (+++). For the rest part of the immunohistochemical markers (Ki-67, p53, and p16), we used quantitative estimation (counting the number of all positive cells in a ×40 objective in 10 fields). The proportion of stained cells was assessed as a percentage (%).

Statistical analysis was performed by using the statistical software package Stata 12 (StataCorp LP, Texas, USA). The Kruskal-Wallis test was used to determine the associations between the different variables. Spearman's test was calculated as the nonparametric correlation coefficient of statistical dependence between two variables. A p value of <0.1 was considered significant.

The Ki-67 antibody recognizes a non-histone protein complex nuclear antigen, which showed only nuclear staining. Our results are contrary to the Mooy et al. data, and did not demonstrate any association with the proportion of the epitheloid cells in tumours or with the high mitotic rate (found with Kruskal—Wallis test p=0,879 and p=0,843, respectively). Moreover, we did not find any correlation between the age of diagnosis and the level of expression marker of cell proliferation (Kruskal—Wallis test p=0,31).

The positive nuclear staining of the p53 was present in 54.7% cases. Unfortunately, we did not find any relationship between p53-level and histological type of UM (Kruskal—Wal-

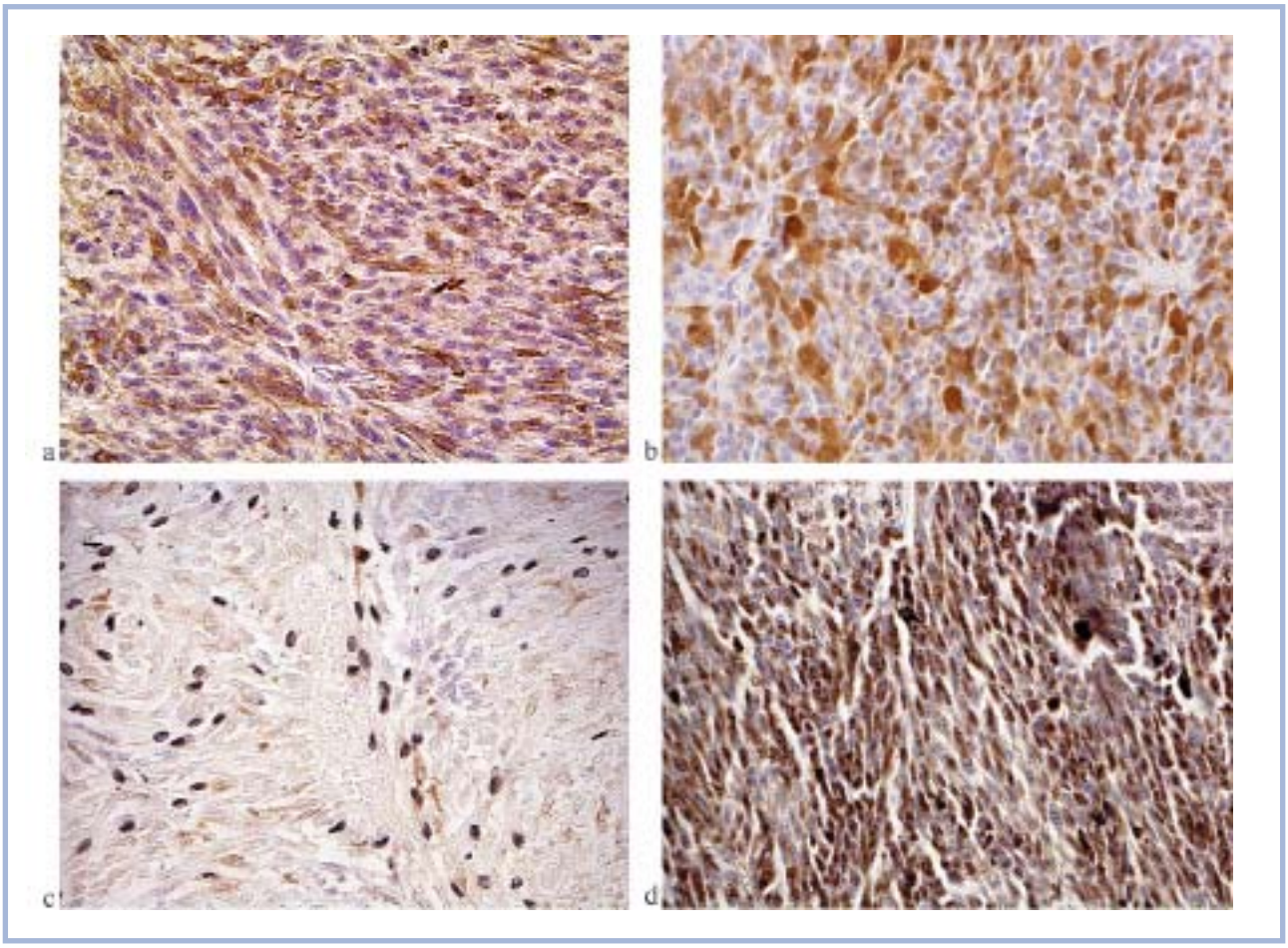

Fig. 1. **Immunohistochemical staining of uveal melanoma section.**

1a. Immunohistochemical positive staining for MMP9 of melanoma section (×400); Diffuse matrix and cytoplasm expression of marker is observed. 1b. P16 showing a variable degree of positive cytoplasm and nuclear staining (×400); 1c. Positive nuclear with low-intensive cytoplasm background expression of TGFb in spindle cells (×400); 1d. EGFR staining of cytoplasm and nuclei in mixed cell melanoma (×400).

lis test p=0,228). Looking more closely at the histological type, there is also no correlation with the presence of epitheloid cells and expression of p53 (Kruskal—Wallis test p=0,812). Another interesting result was, that on the basis of our data, there is no significant association between atypia level and expression of p53 (Kruskal—Wallis test p=0,547).

According to our data the positive staining of the MMP9 was detected in 90.5% of tumours **(Fig. 1a)**. We were looking for the evidence of Y.Y. El-Shabrawi et al.'s theory on the correlation between the level of MMP9-expression and the aggressive histological type of intraocular melanoma [6]. According to our study, MMP9 is predominantly expressed in spindle cell UM (Kruskal—Wallis test p=0,1, **Fig 2a**). Our data do not support the hypothesis noted earlier. Furthermore, there is a tendency to hyperexpression of the MMP9 at the early stages of invasion **(Fig. 2b)**. An interesting result was, that 69% of cases showed positive cell and nuclear staining of the p16 **(Fig. 1b)**. According to our study, the high levels of expression do not correlate with histological type of UM (Spearman's test p=0,9) or mitotic rate (Kruskal—Wallis test p=0,924). On the other hand, the hyperexpression of p16 has a significant association with UM diagnosis at an advanced age (Kruskal—Wallis test p=0,068, **Fig. 3a**). H.J.M. de Jonge et al. suggest that the relationship between the high level of p16 and the diagnosis of malignant tumours in the elderly means there is activity reduction of progenitor haematopoietic stem cells 22. Nevertheless, there is no reliable data to clarify the reason of this phenomenon for UM.

The results of TGFb immunohistochemical reaction revealed a selective nuclear staining for spindle cells **(Fig. 1c)**. According to the recent studies about TGFb pathway in UM, expression of this growth factor has a tendency to up-regulate p16-gene and other cell-cycle inhibitors [17]. Our data reflect that the p16 level reduced in inverse proportion to that of TGFb **(Fig. 3b)**. Moreover, we have revealed the significant correlation between TGFb hyperexpression and atypia level (Spearman's test p=0,059). It is noticeable that the level of TGFb expression increased in inverse ratio to the stage of scleral invasion (Spearman's test p=0,1). Nevertheless, the histological type of UM did not have any impact on the level of TGFb (Kruskal—Wallis test p=0,879).

The positive EGFR-staining was detected in 26.6% of tumours in our research **(Fig. 1d)**. On the one hand, a statistically significant correlation was observed between the high level of EGFR expression and spindle cell type of UM (Kruskal—Wallis test p=0,1). On the other hand, an advanced stage of atypia had no influence on EGFR expression. Nonetheless, according

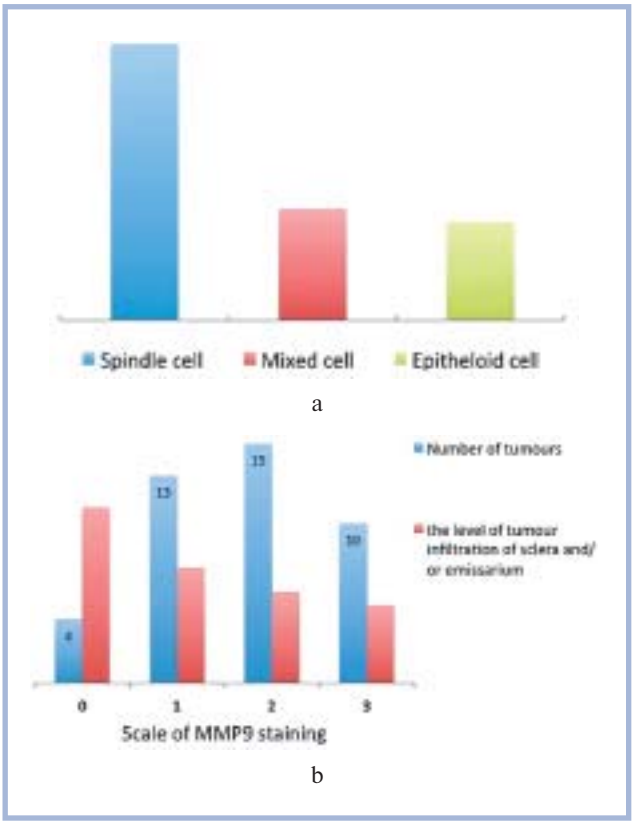

Fig. 2. **MMP9 expression in uveal melanoma.**

2a. MMP9 is predominantly expressed in spindle cell UM; 2b. There is a tendency to hyperexpression of the MMP9 at the early stages of invasion.

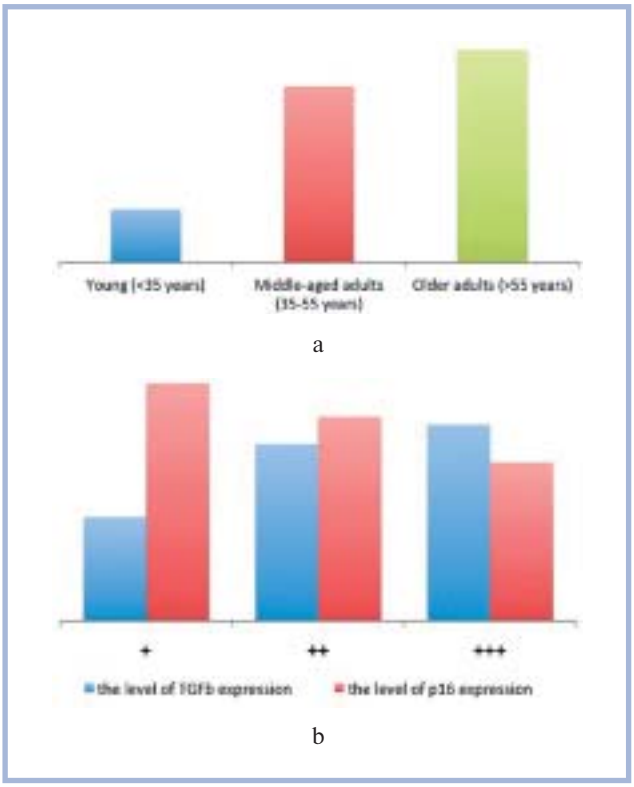

Fig. 3. **P16 expression in uveal melanoma.**

3a. On the other hand, the hyperexpression of p16 has a significant association with UM diagnosis at an advanced age; 3b. The p16 level reduced in inverse proportion to that of TGFb.

to our data, the EGFR hyperexpression correlates with the early stages of scleral infiltration (Spearman's test p=0,038).

The results of the research we have undertaken provided some insights into the pathogenesis of UM. We have found the link between the mitotic rate and the high level of TGFb expression. The latter increased in inverse proportion to the p16 level. As was mentioned earlier, the genetic survey of N. Myatt et al. has provided evidence of mutation (usually deletion) in chromosome 3p22 in most UM, which is also contains the TGF-bR2-gene. Alteration of this receptor lead to disruption of SMAD-mediated signal transduction (e.g. SMAD 2, 3, and 4 types), that resulted in the up-regulation of a number of cdk inhibitors, including p16INK4. Thereupon, we have come to a conclusion about the key-role of abnormalities in TGFb-pathway that cause down-regulation of p16-gene and eventually provoked an increase of mitotic rate. An interesting result is that diagnoses at an advanced age correlate with hyperexpression of p16. On the basis of our data and previous studies, we reached the conclusion that after the lapse of time the level of p16 rises significantly in order to inhibit proliferating activity of melanocytes in the normally functioning pigmented layer. However, although the probability of UM diagnoses in elderly is increasing, we have no reliable data for the relationship with high atypia levels. Thus, p16-pathway needs further genetic approval, subject to the age at diagnosis.

Over the past decade, there has been an increased effort to study the influence of different immunohistochemical markers expression on the histological type of UM. On the basis of our data the hyperexpression of MMP9 and EGFR correlates with a high proportion of spindle cells in a tumour. These results contradict what have been reported before by different authors [5, 6, 20, 23]. EGFR and MMP9, used nowadays as targets for anticancer therapy, have provided a new perspective on oncology. For example, Cetuximab (monoclonal anti-EGFR antibodies) and MiR-885-5p (MiRNA down-regulator MMP9 expression) are new target therapies for colorectal adenocarcinoma and glioblastoma multiforme, respectively [24, 25]. Moreover, we have demonstrated the association between the level of EGFR, TGFb and MMP9 expression and the initial invasion stage. According to our results, it is more convenient to use this type of therapy on the early stages of invasion and the more benign histological type of UM in order to retain an affected eye as an organ.

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