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

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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 — определить иммуногистохимический уровень TGFb, 5p16 и p53, 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 – 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%-формалин 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 проводили с помощью системы En Vision Flex («Dako», Дания) при температуре 95—98 °С и pH 9,0 в течение 20 мин в модуле предобработки к автоматеру (PT-module). В качестве хромогена использовали DAB. Анализ результатов проводился с использованием программы Stata 12 по методу Краскела—Уоллиса, который является многомерным тестом Манна—Уитни для ранговых данных. Для оценки уровня корреляции использовался тест Спирмена, при котором также является ранговым. Уровень значимости (p) принимался равным 0,1 для каждого из методов.

Результаты и обсуждение. В результате исследования подтвердились наличие достоверной корреляции между количеством митозов в высокой экспрессии TGFb (по тесту Спирмена р=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 TFGR2, который в свою очередь ведет к изменениям в SMAD-ассоциатах (а именно SMAD типов 2, 3 и 4), вызывающих синтез каскада ингибиторов сигнала TGFb. 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 TGFb и p53, а также снижение уровня p16 и высокая экспрессия TGFb, paxient age. Key words: uveal melanoma, immunohistochemistry, TGFb, paxient age.
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, TGFβ, EGFR, MMP2 and MMP9 and their role in the pathogenesis of UM. 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-ckd4 and cyclin D-ckd6 kinases and results in cell cycle control at the G1-S restriction point. Recent studies found that the gene CDKN2A (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.

TGFβ 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 TGFβ is produced by the ciliary body and the retina within the eye and that binding to TGFβR1 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].
The information about the primary antibodies

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

Some authors claim that the expression of endoglin (a transmembrane regulatory receptor on proliferating endothelium for TGFβ) 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 (TGFβ 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 radiotherapy. 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 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 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).

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-
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 hyper-expression 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 \( p = 0.01 \)). On the other hand, an advanced stage of atypia had no influence on EGFR expression. Nonetheless, according
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 TGFbR2-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.
REFERENCES


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