Immunohistochemical features of gastrointestinal stromal tumors and the role of expression of p16ink4A, Ki-67, VEGF and MMP-9 in their behavior

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Aim: to clarify the prognostic value of cytoplasmic p16ink4A, VEGF, MMP-9 and Ki-67 expressions in gastrointestinal stromal tumors (GISTs) and connection of different levels of these markers expression with aggressive transformation of GISTs.

Materials and methods. Our study included 36 samples of primary tumors and 10 relapses of GIST and metastases in liver after primary combined treatment (surgery and chemotherapy with imatinib). The immunohistochemical study was performed with 4 primary antibodies: Ki-67, p16ink4A, VEGF and MMP-9. We used formalin fixed and paraffin embedded (FFPE) tissue samples for immunohistochemical study.

Results. In our study we showed significant connection between levels of cytoplasmic expression of p16ink4A in primary GISTs and such markers of tumor aggressive behaviour as Ki-67, MMP-9 and VEGF (Fisher's exact P-value = 0.000753; 0.000101 and 0.000048 respectively).

Between cytoplasmic expression of p16ink4A and VEGF and also between p16ink4A and MMP-9 strong direct correlation was found ($\gamma = 0.829$, $P < 0.05$ and $r_s = 0.961$, $P < 0.05$ respectively). The correlation between expression of Ki-67 and p16ink4A was also direct and strong ($r_s = 0.754$, $P < 0.05$), but with some exclusions, that's why this correlation needs further investigation in larger groups with preciser molecular analysis. Analysis of metastatic GISTs samples showed prominent levels of MMP-9 and VEGF expression.

Conclusions. Our study has shown very important role of cytoplasmic expression of p16ink4A in GIST as one of the markers of aggressive behavior, which can be used in complex with other markers for more accurate prognosis of GISTs progression. Prominent levels of MMP-9 and VEGF expression in metastatic GISTs can be a marker of resistance to imatinib. So probably evaluation of MMP-9 and VEGF expression can be used as a tool for correct choice of chemotherapy for patients with GISTs.

Ключові слова:
гастроінтестинальна стромальна пухлина, цитоплазматична експресія p16ink4A, MMP-9, Ki-67, VEGF, метастази гастроінтестинальної стромальної пухлини, агресивна поведінка гастроінтестинальної стромальної пухлини.

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Імуногістохімічні особливості гастроінтестинальних стромальних пухлин і роль експресії p16ink4A, Ki-67, VEGF і MMP-9 у поведінці цих пухлин

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Мета роботи – з'ясувати прогностичне значення цитоплазматичної експресії p16ink4A та експресії таких маркерів, як VEGF, MMP-9 і Ki-67 при гастроінтестинальних стромальних пухлинах (ГІСП), виявити зв'язок різних рівнів експресії цих маркерів з агресивною поведінкою ГІСП.

Матеріали та методи. Дослідження включало 36 випадків первинних ГІСП і 10 випадків метастазів ГІСП у печінку після первинної комбінованої терапії (хірургічне видалення пухлини та терапія іматинібом), що представлені матеріалом пухлин у парафінових блоках, із них робили зрізи для імуногістохімічного дослідження з маркерами p16ink4A, VEGF, MMP-9 і Ki-67.

Результати. Виявили статистично значущий зв'язок між рівнем цитоплазматичної експресії p16ink4A в первинних ГІСП і такими маркерами агресивної поведінки, як Ki-67, MMP-9 і VEGF (p-значення за точним критерієм Фішера становило 0,000753, 0,000101 і 0,000048 відповідно).

Між цитоплазматичною експресією p16ink4A і VEGF, а також між p16ink4A і MMP-9 встановили сильний прямий кореляційний зв'язок ($\gamma = 0{,}829$, $p < 0{,}05$ та $r_s = 0{,}961$, $p < 0{,}05$ відповідно). Прямий і сильний ($r_s = 0{,}754$, $p < 0{,}05$) кореляційний зв'язок визначили також між експресією Ki-67 і p16ink4A. Аналіз метастатичних ГІСП показав значущу експресію MMP-9 і VEGF.

Висновки. Результати дослідження показали, що рівень експресії p16ink4A корелює з рівнем експресії інших маркерів агресивної поведінки пухлин – Ki-67, MMP-9 і VEGF.

Тому оцінювання експресії p16ink4A можна застосовувати для прогнозування перебігу ГІСП. Значуща експресія MMP-9 і VEGF у метастатичних ГІСП може бути маркером резистентності до іматинібу. Отже, ймовірно, оцінювання експресії MMP-9 і VEGF можна використовувати для корректного призначення хімієтерапії пацієнтам із ГІСП.

Иммуногистохимические особенности гастроинтестинальных стромальных опухолей и роль экспрессии p16ink4A, Ki-67, VEGF и MMP-9 в поведении этих опухолей

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Цель работы – уточнение прогностического значения цитоплазматической экспрессии p16ink4A и экспрессии таких маркеров, как VEGF, MMP-9 и Ki-67 при гастроинтестинальных стромальных опухолях (ГИСО), установление связи между разными уровнями экспрессии этих маркеров и агрессивным поведением ГИСО.

Материалы и методы. Исследование включало 36 случаев первичных ГИСО и 10 случаев метастазов ГИСО в печень после комбинированной терапии (хирургического удаления опухоли и терапии иматинибом), представленных материалом опухолей в парафиновых блоках, из которых в последующем делали срезы для иммуногистохимического исследования с маркерами p16ink4A, VEGF, MMP-9 и Ki-67.

Результаты. Установлена статистически значимая связь между уровнем цитоплазматической экспрессии p16ink4A в первичных ГИСО и такими маркерами агрессивного поведения опухолей, как Ki-67, MMP-9 и VEGF (p-значение точного критерия Фишера составило 0,000753, 0,000101 и 0,000048 соответственно).

Между цитоплазматической экспрессией p16ink4A и VEGF, а также между p16ink4A и MMP-9 установлена сильная прямая корреляционная связь ($\gamma = 0,829$, $p < 0,05$ и $r_s = 0,961$, $p < 0,05$ соответственно). Корреляционная связь между экспрессией Ki-67 и p16ink4A также прямая и сильная ($r_s = 0,754$, $p < 0,05$). Анализ метастатических ГИСО выявил значительную экспрессию MMP-9 и VEGF.

Выводы. Результаты исследования показали, что уровень экспрессии p16ink4A коррелирует с уровнем экспрессии других маркеров агрессивного поведения опухолей – Ki-67, MMP-9 и VEGF. Поэтому оценка экспрессии p16ink4A может быть использована для прогноза течения ГИСО. Выраженная экспрессия MMP-9 и VEGF в метастатических ГИСО может быть маркером резистентности к иматинибу. Поэтому, вероятно, оценку экспрессии MMP-9 и VEGF можно использовать для корректного назначения химиотерапии пациентам с ГИСО.

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of digestive tract [1]. These tumors have variable behavior; many of them are characterized by indolent course. However, at the same time, there are too less histological criteria, that allow to predict progression of GISTs. Criteria that were used in last histological classification of digestive system tumors include mitotic rate, tumor size and tumor site of location and only 1st criterion in this list is histological. Probably due to this fact all GISTs in last classification are coded as malignant tumors (8936/3) [1,2].

Immunohistochemical analysis is a useful tool in routine pathomorphological practice that helps to put more accurate diagnosis and choose correct treatment for patients. There is a specter of immunohistochemical markers that shows the aggressive potential of different tumors, such as the ability of a tumor to invasion (MMP-9), angiogenesis (VEGF) and proliferative activity (Ki-67). In different studies, there were shown effectiveness of Ki-67, VEGF and MMP-9 as powerful predictive markers of GISTs outcome, but there are no studies, that investigated the role of these markers in tumor progression in complex.

In research paper from 2014 it was shown, that Ki-67 index more than 8 % can be an exact factor of worse prognosis and poor response for imatinib treatment for patients with primary GISTs [3]. Naiqing Liu et al. in their study of 52 primary GISTs show that the expression levels of MMP-9 and VEGF were significantly higher in malignant GIST than that in benign GIST. Their expression levels were associated with the GIST tumor size, invasion and metastasis, mitotic count and central necrosis [4,5].

The situation with such a marker as p16ink4A is more ambiguous. P16ink4a is a particularly potent effector of cell cycle progression that functions in concert with CDK4/Cyclin D and RB in coordinating proliferation. Application of immunohistochemical analysis with p16ink4A can be used as a novel biomarker for the detection of cancer cells in early stages [6]. The high-level expression of p16ink4a in tumors is associated with aggressive subtypes of disease, and in certain clinical settings elevated p16ink4a expression is an important determinant for disease prognosis and therapeutic response [6–8]. Some authors have shown that there are 2 patterns of p16ink4A expression in GISTs: nuclear and cytoplasmic. Each of these patterns plays an independent role in progression of GISTs. Haller et al. in their study confirmed that the low nuclear expression of p16ink4a and high cytoplasmic expression of p16ink4A in tumor cells are independently associated with progression in GISTs [7]. But in their study they also said that for the quality of nuclear expression of p16ink4a can influence different factors, such as the age of individual tumor samples, in contrast to it cytoplasmic expression was more stable and non-tumor cells had never shown cytoplasmic expression of p16ink4a [7]. Other authors have shown that the nuclear expression of p16ink4a with or without cytoplasmic expression was higher in GISTs with the shortest recurrence free period [9,10]. This data contradicts partially to Haller's results, because told about the role of high nuclear expression in tumor progression. However, if to study this data more careful, we can see that authors didn't mention what percentage of tumors in their group have cytoplasmic expression of p16ink4a, that's why we are not able to totally exclude role of cytoplasmic expression of p16ink4a in aggressive behavior of GISTs.

There are also no complex studies, that have shown the role of p16ink4A, Ki-67, VEGF and MMP-9 expression not only in primary GISTs, but also in their metastases. However, this information will be very useful not only for understanding the biological characteristics of metastases of GISTs, but also for evaluation of effectiveness of treatment and for choosing the better strategy of therapy.

Aim

To investigate aggressive potential of primary GISTs by examining expression of Ki-67, MMP-9, VEGF and p16ink4A in these tumors. In addition, to study the levels of expression of these markers in metastases of GISTs obtained from patients who underwent chemotherapy with imatinib. Since p16ink4A is not a popular marker of aggressive potential, but it plays a crucial role in oncogenesis, we put as an aim of this research to determine the role of different levels of cytoplasmic expression of p16ink4A in GIST behavior by comparing expression of this marker with expression of well-studied markers of tumor proliferative activity (Ki-67), invasiveness (MMP-9) and neoangiogenesis (VEGF).

Materials and methods

The study was performed on formalin fixed and paraffin embedded (FFPE) tumor samples of GISTs. The material included tumor samples of primary GISTs obtained from 36 patients, who have undergone surgical excision of tumors and tumor samples of metastases of GISTs in liver from 10 patients, who were treated with imatinib after surgical excision of primary tumor. Postoperative tumor material was obtained from pathology departments of "Grigoriev Institute for Medical Radiology and Oncology of the National Academy of Medical Sciences of Ukraine", Kharkiv, Ukraine and "National Cancer Institute", Kyiv, Ukraine. All surgical resections were performed between 2016 and 2019.

Eligibility criteria included the availability of follow-up data at least for a year after surgical resection of primary tumor and information about treatment for patients with metastases, good quality and sufficient quantity of tumor material for immunohistochemical analysis, positive immunohistochemical staining with CD117 and DOG-1 confirming diagnosis GIST. We divided primary tumor samples by location on gastric and non-gastric sites and by size into 4 groups: ≤2 cm; >2 cm to ≤5 cm; >5 cm to ≤10 cm and >10 cm. Such a subdivision was based on prognostic parameters given by United States Armed Forces Institute of Pathology (AFIP) and recommended in last WHO classification of digestive system tumors (2019) [1].

The immunohistochemical study was performed with 4 primary antibodies: Ki-67, p16ink4A, VEGF and MMP-9. The characteristics of these antibodies are given in *Table 1*. We used formalin fixed and paraffin embedded (FFPE) tissue samples for immunohistochemical study. The whole study was performed according to the Dako protocol for manual IHC staining.

Quantitive method was used for evaluation of Ki-67, MMP-9 and p16ink4A expression.

Ki-67 score was expressed as the percentage of the number of immunopositive nuclei among the total number of nuclei of tumor cells regardless of the immunostaining intensity. The counting was performed in three areas of most intensive staining ("hot spot" areas) at ×400 magnification. The expression of proliferation index Ki-67 is categorized into 3 groups: ≤5 % positive cells, 6–8 % positive cells and >8 % positive cells according to cutoff levels given in Wen-Yi Zhao and coauthors study of 418 GISTs in 2014 [3].

MMP-9 expression was also evaluated with use of quantitative method and then divided into groups: score 0 – negative staining with MMP-9, score 1 – percentage of positive cells less than 30 %, score 2 – percentage of positive cells between 30 % and 70 %, score 3 – percentage of positive cells more than 70 % [5].

Expression of p16ink4A was calculated as the percentage of the number of immunopositive cells (positive cytoplasmic staining with or without nuclear staining) among the total number of tumor cells in three areas of most intensive staining (in cases of heterogeneous expression) at ×400 magnification. Then all cases were subdivided into groups according to Remmele and Stegner method [11] and important cutoff value at 10 %: score 0 (-) – negative staining with p16ink4A, score 1 (+) – 1 % to 9 % of positive cells, score 2 (++) – 10 % to 49 %, score 3 (+++) – 50 % and more positive cells. We analyzed 2 important groups in our study: negative or low (+) expression of p16ink4A compared to moderate (++) or high (+++) expression of p16ink4A.

The semiquantitative approach was used for the assessment of VEGF expression. The granular cytoplasmic expression of VEGF was scored in such a scale: score 0 (-) – no expression, score 1 (+) – weak positive staining, score 2 (++) – positive staining, score 3 (+++) – strong positive staining [5].

Results were visualized and photographed using light microscope (ZEISS Primo Star, ZEISS Axiocam ERc5).

The relationship between different clinicopathological parameters of tumors were estimated using the Fisher's exact test (Fisher's exact P-value < 0.05). Spearman's rank correlation and Gamma coefficients were used for the measurement of rank correlation between quantitative variables and between quantitative and semiquantitative variables, respectively. An extensive parameter (%) was used to describe qualitative characteristics. All statistical analyses were performed using Microsoft Excel 2013 and MedCalc.

The design of the study and all the methods used in the study were approved by the Bioethics Committee of the above institutions and complied with the requirements of the Declaration of Helsinki. Such clinicomorphological criteria as gender, site of tumor and its size were obtained from case histories, where we analyzed surgical protocols, data about chemotherapy and data from histological conclusions.

Results

Primary GISTs. We divided primary GISTs into 2 groups using as a cutoff value 10 % of p16ink4A positive cells in tumor sample. The first group included 19 patients with score 0 (-) or score 1 expression of p16ink4A. The second group consists of 17 patients with expression of p16ink4A ≥10 % (score 2 and 3). The aim of such subdivision was to find the relationship between different clinicopathological parameters of GISTs and p16ink4A expression. Gender of study objects was well matched. Some parameters as tumor location, size, proliferative index, expression of MMP-9 and VEGF according to multiple data play role in risk of tumor progression. All data that we get are shown in *Table 2*. As we see from this table the statistically signi-

ficant results with P exact value < 0.001 were given only for Ki-67 expression and p16ink4A, for VEGF expression and p16ink4A, for MMP-9 expression and p16ink4A.

Spearman's rank correlation coefficient was used to show exact relationship between p16ink4A expresion and other immunohistochemical parameters of tumor aggressive behavior (Ki-67, MMP-9). Gamma coefficient was used for description of relationships between semiquantitive parameters as VEGF expression and quantitative variables such as expression of p16ink4A.

We obtained the following results. The direct very strong relationship according to Chedock's scale was found between expression of MMP-9 and expression of p16ink4A in primary GISTs (r_s = 0.961, P < 0.05). This result means that with the elevation of MMP-9 expression the expression of p16ink4A will also rise.

The direct strong relationship according to Chedock's scale was found between expression of VEGF and p16ink4A in primary GISTs (γ = 0.829, P < 0.05).

Almost the same results were obtained between expression of Ki-67 and p16ink4A (r_s = 0.754, P < 0.05), but here relationship was strong compared with very strong relationships in previous calculations. Additionally, we have to discribe some cases those were not typical for general results. We had one case with quite high expression of Ki-67 (38 %), but low expression of p16ink4A (8 %). Expression of VEGF in this case was moderate (++) and expression of MMP-9 was 50 %. But this tumor had size of 6.5 cm and was localized in stomach, so using prognostic criteria given in WHO histological classification of digestive system tumors [1] risk of progression for this tumor is 55 % compared to 85 % for similar tumor with intestinal location. The next 2 cases were in group with Ki-67 ≤8 %. These tumors had high expression of p16ink4A (31 % and 95 %, respectively). Both tumors are localized in small intestine, their size were less than 5cm, expression of MMP-9 were 15 % and 78 %, respectively. So if we take into account all clinicopathological features of these tumors, we will see that despite of low Ki-67, expression of MMP-9 and tumor location besides of expression of p16ink4A can show that these tumors have quite prominent risk of tumor progression.

Metastatic GISTs. For group of metastatic GISTs we got following results. The expression of all markers in metastatic GISTs were heterogenous with areas of higher expression and with areas where expression was quite low. The mean expression of Ki-67 was 8.4 ± 2.9 %. All metastatic GISTs in our study had p16ink4A expression lower than 10 % (5.2 ± 2.7 %). At the same time, expression of VEGF and MMP-9 were quite prominent. 6 GISTs among 10 showed strong positive staining with VEGF (+++) and 4 GISTs (4/10) had moderate expression of VEGF (++). The levels of MMP-9 expressions were very variable. The mean expression of MMP-9 in metastatic GISTs was 38 ± 30 %. Due to small amount of cases in group of metastatic GISTs we didn't make any correlation analysis here.

Discussion

In our study we have shown the direct strong relationship between moderate and high expression of p16ink4A and

Table 1. List of primary antibodies used in our study

Primary antibody	Clon	Dilution	Manufacturer
MMP-9	Ab-1 GE-213	1:200	ThermoFisher Scientific, USA
Ki-67	SP6	1:400	ThermoFisher Scientific, USA
VEGF	JH121	1:20	ThermoFisher Scientific, USA
P16ink4A	1D7D2	1:200	ThermoFisher Scientific, USA

Table 2. Clinicopathological features of primary GISTs and their relationships with cytoplasmic p16ink4A expression

Clinicopathological characteristics	p16ink4A cytoplasmic staining (-/+) (number of cases)	p16ink4A cytoplasmic staining (++/+++) (number of cases)	P value
Gender			
Male	11	7	0.34311
Female	8	10	
Tumor site			
Stomach	8	3	0.15596
Non-gastric site	11	14	
Tumor size (cm)			
≤2	3	1	0.138
>2 to ≤5	10	7	
>5 to ≤10	6	5	
>10	0	4	
Ki-67 expression (%)			
≤5	11	2	0.000753*
6-8	4	0	
>8	4	15	
VEGF expression			
-/+	15	1	0.000048*
++	4	6	
+++	0	10	
MMP-9 expression			
Score 0	11	0	0.000101*
Score 1	7	2	
Score 2	1	10	
Score 3	0	5	

*: P-value <0.001 (Fisher's exact test).

important markers of aggressive potential of tumors, such as Ki-67 (proliferative index), MMP-9 (invasive capacity) and VEGF (the marker of angiogenesis in tumors). Recent data has shown that expression of cell cycle regulators such as cyclin D1, CDK4 and p16ink4A is associated with high risk of GIST recurrence [12,13]. Shaffer and coauthors in their paper demonstrated significant association between strong and diffuse p16 protein expression in GISTs and loss of MAX or p16ink4A coding sequence mutations. At the same paper they said that inactivation of MAX gene is a common event in GIST progression [14].

The investigation of cytoplasmic expression of p16ink4A in GISTs was one of our main issues, because the role of cytoplasmic expression in GISTs is not enough elucidated in scientific papers. Only F. Haller et al. in their article described the cytoplasmic expression of p16ink4A as an independent factor of worse prognosis in GISTs [7]. Other authors studied only nuclear expression of p16ink4A in GISTs [9,10] and very often did not take into account the presence or absence of cytoplasmic expression in their cases [10]. However, the independent role of cytoplasmic expression of p16ink4A as a factor of poor prognosis were described in such tumors as laryngeal

squamous cell carcinomas (SCC) [11], neuroendocrine tumors of digestive system [15] and concurrent nuclear and cytoplasmic overexpression of p16ink4A in diffuse gliomas was associated with a worse outcome [16]. Moreover, the latest data demonstrate, that cytoplasmic p16ink4A is not an artefact and can be involved in the dissociation of focal adhesions, and then related to the cell invasion [8]. Our finding supports this position to show direct strong correlation between levels of MMP-9 and p16ink4A in tumor cells. Additionally, S. Mendaza et al. found relationship between cytoplasmic p16 and Angiotensin II receptor associated protein (AGRAP) [8] that can increase expression of VEGF in tumor cells [17] and due to this pathway stimulate angiogenesis and tumor growth. In our study, we found direct strong correlation between expression of p16ink4A and VEGF. It is also important to mention, that expression of MMP-9 and VEGF are closely related probably due to influence of epidermal growth factor receptor (EGFR) on their production [18]. That is why to discover the molecular connections between cytoplasmic p16ink4A, MMP-9 and VEGF can be an issue of many future studies.

The study of laryngeal squamous cell carcinomas has shown similar association between Ki-67 expression and cytoplasmic p16ink4A, as we got in our study [11]. The expression of cytoplasmic p16 ink4A increased in laryngeal SCC with increasing of tumor grade [11]. But as we saw in some our cases, not always we can see strong correlation between Ki-67 and p16ink4A, that's why expression of these markers has to be analyzed in combination with other clinical and pathological factors of tumor progression. Moreover, additional molecular studies are needed to clarify the connection between Ki-67 and p16ink4A expression.

The metastatic GISTs have shown ambiguous results, from one side they had quite low levels of Ki-67 and p16ink4a expression, from the other side expression of VEGF and MMP-9 were prominent. Probably such results are associated with small number of cases in this group or it can also be a feature of chemotherapeutic effects, because all patients were treated with imatinib. Anyway, it has to be mentioned, that different scientific papers point out that high VEGF expression is associated with low therapeutic response to imatinib and as a result leads to tumor progression [19–21]. We observed in the group of metastatic GISTs quite high expression of VEGF, that probably can be a marker of resistance to imatinib and for those patients therapy with sunatinib is highly recommended. Additionally, we have found the study demonstrating loss of sensitivity to imatinib in p16-deleted metastatic GISTs [22]. Hence, low levels of p16ink4A expression in metastatic GISTs in our study might be a sign of such resistance.

Conclusions

1. Our study has shown a very important role of cytoplasmic expression of p16ink4A in GIST as one of the markers of aggressive behaviour, which can be used in complex with other markers for a more accurate prognosis of GISTs progression. We showed significant connection between levels of cytoplasmic expression of p16ink4A in primary GISTs and such markers of tumor aggressive behaviour as Ki-67, MMP-9 and VEGF (Fisher's exact P-value = 0.000753; 0.000101 and 0.000048, respectively). Between cytoplasmic expression of p16ink4A and VEGF and also between p16ink4A and MMP-9 strong direct correlation was found ($\gamma = 0.829$, $P < 0.05$ and $r_s = 0.961$, $P < 0.05$ respectively). The correlation between expression of Ki-67 and p16ink4A was also direct and strong ($r_s = 0.754$, $P < 0.05$), but with some exclusions, that's why this correlation needs further investigation in larger groups with preciser molecular analysis.

2. Analysis of metastatic GISTs samples showed prominent levels of MMP-9 and VEGF expression, that can be a marker of resistance to imatinib. So probably evaluation of MMP-9 and VEGF expression can be used as a tool for correct choice of chemotherapy for patients with GISTs. Our study of metastatic GISTs has some limitations due to small number of tumors in this group, that's why further studies in larger groups are needed.

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