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NEOANGIOGENESIS IN THE DYNAMICS OF TESTICULAR SEMINOMA PROGRESSION

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Abstract

Testicular germ cell tumors (TGCT) is a group of neoplasms which develop from the germ cell epithelium and account 94-96% of all testicular tumors. Seminoma is the most common testicular germ cell neoplasm and accounts for about 50% of all TGCT.

There is an increasing evidence that metastatic disease in both early and late stages depends on the degree of tumor vascularization. But despite the large number of publications on this subject, the ways of new vessels formation in invasive neoplasms and effect of the intratumoral vessels density on overall and relapse-free survival rate remain unclear.

The performed investigation of neoangiogenesis in the dynamics of testicular seminoma progression revealed: significant increasing of relative area of CD31 and CD34 expression as well as vascular density during transition from the initial to the late stages of tumor progression; formation of intratumoral vessels in the seminoma occurs by angiogenesis and vasculogenesis with participation of progenitor endothelial cells; seminoma is characterized by vasculogenic mimicry in the form of channels formation that do not have endothelial lining.

Key words: testicular germ cell tumors; seminoma; angiogenesis; immunohistochemical investigation.

Testicular germ cell tumors (TGCT) is a group of neoplasms which develop from the germ cell epithelium and account 94-96% of all testicular tumors [1]. Seminoma is the most common testicular germ cell neoplasm and accounts for about 50% of all TGCT [2].

Nowadays, a wide range of molecular biological markers are used for immunohistochemical (IHC) analysis of tumors. Evaluation of angiogenesis in the tumor is considered to be one of the markers of prognosis of the disease course, presence of metastases and sensitivity to antitumor therapy. There is increasing evidence that metastatic disease in both early and late stages depends on the degree of tumor vascularization. Conducted researches confirm the importance of tumor angiogenesis as an independent prognostic marker [3].

However, despite the large number of publications on this subject, the ways of new vessels formation in invasive neoplasms and effect of the intratumoral vessels density on overall and relapse-free survival rate remain unclear. And there are very few works in which retraction clefts in tumorous tissue are studied [4].

CD31 and CD34 are highly sensitive biological markers for the differentiation of endothelial cells (EC) which are widely studied in tumorous angiogenesis [5-7].

At the same time, data concerning the study of the mechanisms of blood vessels formation, the work out of methods for estimation of tumorous angiogenesis as well as the use habits of IHC markers of EC (CD31 and CD34) for assessing the aggressiveness and prognosis of TGCT and, in particular, seminomas, are absent in the available literature sources.

Objectives: to study peculiarities of neoangiogenesis in the dynamics of testicular seminoma progression.

Material and methods of study. In the furtherance of this goal, examination of surgically removed seminal gland was carried out and analysis of medical case histories of 13 patients with seminoma was made. These patients were examined and treated in Kharkiv Regional Clinical Centre of Urology and Nephrology named after Shapoval V.I. The investigation covered period from 1998 to 2017.

All the investigated observations were distributed in accordance with pathological pTNM classification [2], which is highly important, as the precise diagnosis and staging, which is made in compliance with the advanced science, are fundamental [8].

For the most demonstrative comparison of IHC characteristics, all the studied seminomas were divided according to the degree of tumorous progression.

Thus, guided by the pTNM classification, the following groups were formed:

1. Group «1»: Tumor limited to testis and epididymis, without vascular / lymphatic invasion; tumor may invade tunica albuginea but not tunica vaginalis; no regional and distant lymph node metastasis; serum tumor markers had different levels. Tumors of this group corresponded to the stages $T_1N_0S_{0-2}$.

2. Group «2»: Tumor limited to testis and epididymis with vascular / lymphatic invasion, or tumor extending through tunica albuginea with involvement of tunica vaginalis; presence of lymph node but no distant metastasis; serum tumor markers had different levels. Tumors of this group corresponded to the stages $T_2N_{1-3}S_{0-2}$.

3. Group «3»: Tumor invades spermatic cord, with or without vascular / lymphatic invasion; presence of lymph node but no distant metastasis; serum tumor markers had different levels. Tumors of this group corresponded to the stages $T_3N_{1-3}S_{0-2}$.

4. Group «4»: presence of distant metastasis; serum tumor markers had different levels. Tumors of this group corresponded to the stages $T_{2-3}N_{0-3}S_{0-2}$.

The material for IHC investigation was fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin. From the prepared blocks serial sections thick 4×10^{-6} m were made, then plotted on high-adhesive slides «SUPER FROST PLUS» («DAKO», Denmark) and dried at a temperature 37°C for 18 hours. Disclosure was performed by boiling of sections in citrate buffer (pH 6,0). For visualization of primary antibodies, the Ultra Vision Quanto Detection Systems HRP Polymer (Thermo Fisher Scientific Inc., USA) was used. DAB (diaminobenzidine) was used as a chromogen. Slides were stained with Mayer's hematoxylin and enclosed in Canadian balsam. For each marker in order to exclude false-positive or erroneous results control researches were performed with use of sections of tissues recommended by the antibody producer for a positive control. In addition, for each case negative control without application of primary antibodies was made.

For estimation of neoangiogenesis and degree of vascularization in the dynamics of testicular seminoma progression Mo a-Hu CD31 Monoclonal Antibody, Clone JC/70A («Thermo Fisher Scientific Inc.», USA) and Mo a-Hu CD34 Monoclonal Antibody, Clone QBEND/10 («Thermo Fisher Scientific Inc.», USA) were used.

To achieve a high-quality and objective analysis of digital images a technique that made it possible to process images with maximum efficiency and obtain more accurate and informative quantitative data was developed [9].

Stained sections of tumor tissue were registered using microscope «Olympus BX-41TF» (Japan) and digital camera «Olympus C3040-ADU» (Japan). The received photos were processed in the Matlab software package using standard digital image processing tools.

For morphometric measurements of the relative area (S) occupied by the immunopositive structures determined in % was automatically calculated in the selected area. Using the brightness values of the RGB color channels in each pixel of the original image the auxiliary color coordinates of the CIE XYZ were calculated and then the color coordinates of the CIE Lab were calculated. Thus, the output digital image corresponded to a three-dimensional array of CIE Lab color coordinates. One of them is lightness / intensity (L), the value of which ranged from 0 to 100. At that L=0-40 corresponded to a strong, L=40-50 to medium, and L=50-100 to a weak level of marker expression intensity. S and L of markers expression was studied in 10-30 randomly selected fields of view of the microscope «Olympus BX-41TF» (Japan) at magnification ×200 ($3{,}12\times10^{-7}$ m^2) in each observation.

Vascular density (VD) as a reflection of the degree of vascularization was determined by counting the number of microvessels in the standardized field of vision (SFV) of microscope «Olympus BX-41TF» (Japan) at magnification ×200 ($3{,}12\times10^{-7}$ m^2). For identification of microvessels endothelial cell marker Mo a-Hu CD34 Monoclonal Antibody, Clone QBEND/10 («Thermo Fisher Scientific Inc.», USA) was used. In each observation 20 SFV were analyzed.

Statistical data processing was carried out using the statistical analysis package of the trial version «STATISTICA 13.3 EN». Comparison of the central trends was performed using Mann-Whitney test for statistical analysis [10, 11] because the sample volume in the groups did not exceed 5 observations. However, descriptive statistics are traditionally presented as mean ± error of the mean (M±m) as long as it is difficult to determine the median and quartiles for a sample of 4-5 observations. The accepted level of significance was $p\leq0{,}05$. Spearman's rank correlation coefficient (r) was counted for measure of the strength of relationship between paired data [10].

Results and discussion. Quantitative indicators of CD34 and CD31 expression which reflect the state of vascularization in the dynamics of testicular seminoma progression are presented in table 1.

The analysis of CD34 showed that in the seminoma the differences in S of its expression differed only between the later stages of tumor progression, not having a difference in this parameter with the initial stage. Thus, in group «1», the average S of the CD34-positive staining was insignificant, and L was on the boundary between strong and moderate level. In the tumor tissue, small and, to a lesser extent, medium caliber vessels prevailed, in which EC with membrane and cytoplasmic staining were well visualized.

Table 1

Indicators of vascularization in the dynamics of testicular seminoma progression

	Group «1» (n_1=4)	Group «2» (n_2=5)	Group «3» (n_3=3)	Group «4» (n_4=1)	p
CD34, S, %	1,79±0,11	1,81±0,03	1,92±0,02	2,01±0,05	p_{12}>0,05 p_{13}>0,05 p_{23}=0,036
CD34, L, unit.	39,9±0,3	39,54±0,19	39,21±0,22	39,12±0,11	p_{12}>0,05 p_{13}>0,05 p_{23}>0,05
CD31, S, %	1,63±0,04	1,85±0,05	1,95±0,04	2,05±0,02	p_{12}=0,016 p_{13}=0,034 p_{23}>0,05
CD31, L, unit.	39,39±0,17	39,25±0,27	39,34±0,17	39,19±0,07	p_{12}>0,05 p_{13}>0,05 p_{23}>0,05
VD, number of vessels in SFV	45,05±2,37	53,59±0,77	60,27±0,61	70,65±0,77	p_{12}=0,016 p_{13}=0,034 p_{23}=0,025

Neoangiogenesis in the seminoma occurred by migration and proliferation of differentiated EC from preexisting blood vessels. In the sections of the neogenic capillaries, which looked like round or tubular structures, CD34 immunopositive EC were visualized. Also, single or small clusters and chains of CD34-positive progenitor EC involved in vasculogenesis were determined in tumor tissue (Fig. 1, A, B).

In group «2» the average S of CD34-positive staining and L of its expression did not differ from similar indicators in group «1». The processes of angio- and vasculogenesis occurred by analogy with the previous group, but progenitor EC were determined more rarely, what testifies the predominance of angiogenesis at this stage.

In group «3» the average S of CD34 expression was higher than that in group «2», but did not differ (p>0,05) from the corresponding indicator of group «1». L of CD34 expression corresponded to a strong level and also did not differ (p>0,05) from that in groups «1» and «2».

In single observation of group «4» the average S of CD34 expression was one of the highest among all studied seminomas, and the L expression of the marker was strong. Visually, as in the previous groups, distribution of blood vessels in the tumor was non-uniform (Fig. 1, C).

At analysis of the data of IHC investigation of CD31 it was found that S of its expression in seminoma was increasing during transition from the initial to later stages of tumor progression.

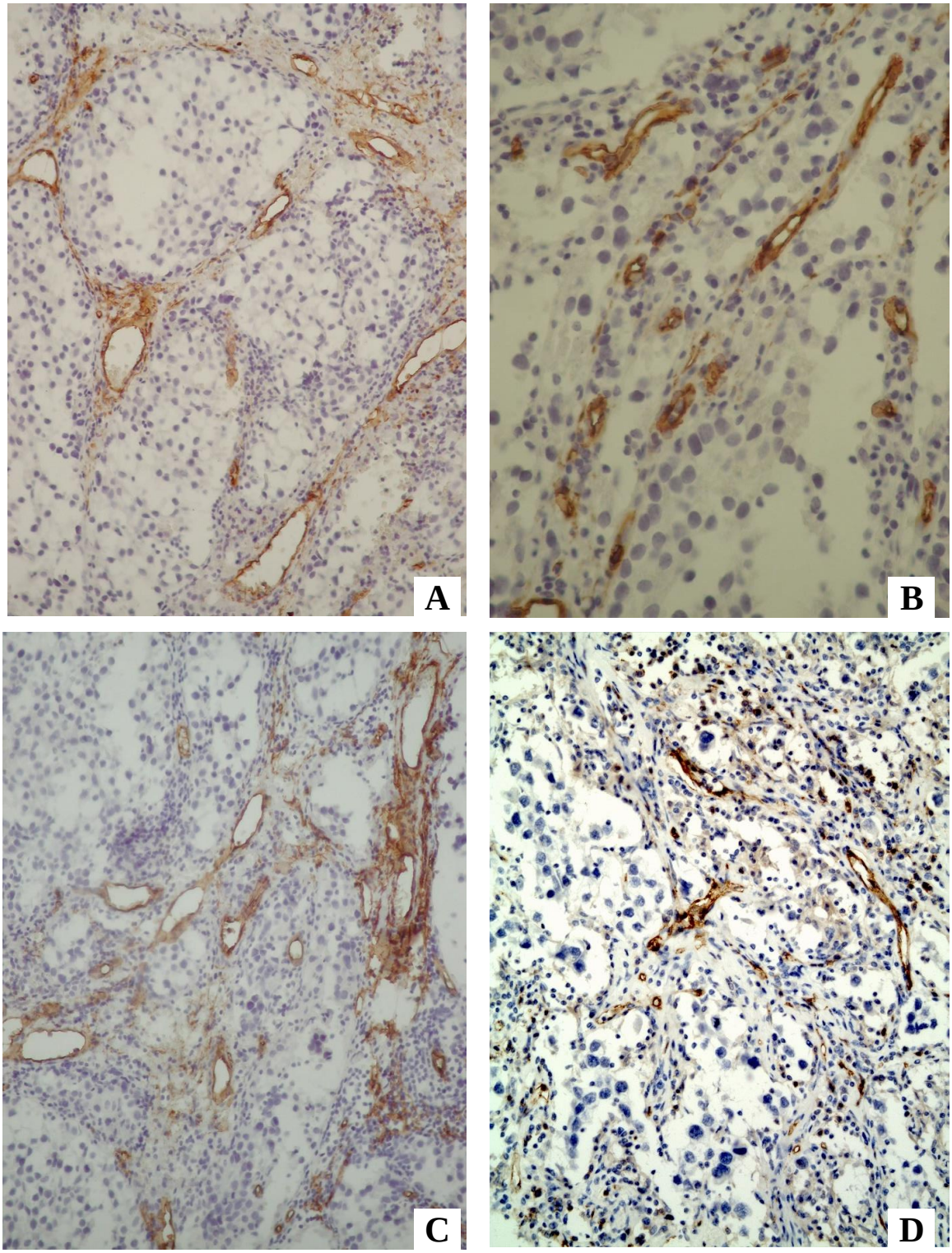

Fig. 1. A. Intensive reaction with CD34 in the vessels of seminoma of group «1» (×200). B. Intensive reaction with CD34 in the vessels and cytoplasm of progenitor EC in seminoma of group «1» (×400). C. Intensive reaction with CD34 in the vessels of seminoma of group «4» (×200). D. Intensive reaction with CD31 in the vessels of seminoma of group «2» (×200). Immunoperoxidase reaction.

Thus, S of CD31-positive staining in group «1» was insignificant, and L – strong. Medium and small caliber vessels were detected in the tumor with predominance of the latter, in which EC with membrane-cytoplasmic staining were visualized. Neogenesis of microvessels occurred mainly by way of angiogenesis: in the walls of the «maternal» vessels lateral outgrowths in the form of cords with EC (in which gaps were sometimes observed) were detected. In group «2» the average S of CD31-positive staining was higher in comparison with that in group «1» and the level of L was strong and did not differ from the same indicator in the previous group. The processes of angiogenesis occurred by analogy with the group described above (Fig. 1, D). Visually, as in the previous groups, distribution of blood vessels in the tumor was non-uniform.

Thus, S of CD31-positive staining in group «1» was significantly lower in comparison with that in the later stages (groups «2» and «3»), and S of CD31 expression in the only case of group «4» was higher than similar indicator in each observation in groups «1» and «2» ($p<0,05$). At the same time, there were no significant differences in S of CD31 expression between groups «2» and «3». L of CD31 expression in seminoma was strong, and its level did not have differences between the studied groups.

Another step in the investigation of the intratumoral vascular component was the determination of VD in the SFV. This method characterizes angiogenic activity and therefore can be used as a marker of transition from the pre-invasive to invasive stage of the primary tumor [12, 13] giving a concept of the difference in blood vessels distribution in different parts of the tumor. In addition, this method allows to divide patients according to the degree of angiogenic activity of the tumor, what is important at prescribing to patients target antiangiogenic therapy [14]. Increasing of VD is considered as unfavorable factor for many tumors [15, 16].

Visually VD in different parts of the seminoma looked non-uniform. A morphometric study found that VD in seminoma was increasing during transition from the initial to later stages of tumor progression having significant differences in the mentioned parameter in each of the studied groups (with the exception of group «4» which was represented by only one observation).

For a long time neoangiogenesis was considered as the only way to deliver nutrients and oxygen to the tumor. In recent years alternative mechanisms of tumor blood supplying have been identified. Formation of vascular channels limited by the basement membrane but in the absence of EC and fibroblasts is called «vasculogenic mimicry» [17]. It has been hypothesized that vasculogenic mimicry is the same organic component of the biology of malignant tumor as well

as inactivation of apoptosis, genomic instability, escape from immune response, induction of angiogenesis and ability to metastasize [18].

In the seminoma, in addition to the processes of neovascularization, we observed the phenomena of vasculogenic mimicry – formation of channels lined with tumor cells through which blood passes. Unlike vessels these channels did not have a lining with EC and, accordingly, they did not have CD34-immunopositive staining.

At evaluation of CD34 and CD31 numerical indicators using Spearman's nonparametric correlation coefficient it was observed very high and moderate positive associations between S of CD31 and CD34 expression with VD during tumorous progression ($r= +0.93$; $r= +0.56$; $p<0,05$, respectively).

Thus, the correlation analysis in combination with the mentioned above data demonstrated an increase of neoangiogenesis in seminoma at tumor progression and also confirmed the effectiveness of the applied method for calculating of S of CD31 and CD34expression.

It was also found that in patients with lymphogenic metastases vascular invasion as well as invasion of the epididymis, tunica albuginea and tunica vaginalis VD and S of CD31 expression were significantly higher than in patients with no specified characteristics. In addition, at comparison of IHC parameters in patients with invasion of the epididymis and patients without it, it was found that L of CD34 expression was significantly lower in the last. In patients with seminoma, who had invasion of spermatic cord, VD was significantly higher than in patients without it (table 2).

Mentioned above proves that development of clinical and morphological signs of seminoma aggressiveness such as invasive growth and development of metastases are mediated by increasing of parameters of CD31 and CD34 expression, reflecting the processes of neoangiogenesis and degree of vascularization.

Table 2

Association of IHC parameters with seminoma aggressiveness

Mann-Whitney test						
		CD31, S, %	CD31, L, unit.	CD34, S, %	CD34, L, unit.	VD, number of vessels in SFV
Lymphogenic metastases	«+»	9	9	9	9	9
	«–»	4	4	4	4	4
	p	0,01	>0,05	>0,05	>0,05	0,01
Vascular invasion	«+»	9	9	9	9	9
	«–»	4	4	4	4	4
	p	0,01	>0,05	>0,05	>0,05	0,01
Invasion of epididymis	«+»	5	5	5	5	5
	«–»	8	8	8	8	8
	p	0,01	>0,05	>0,05	0,02	0,04
Invasion of tunica albuginea and tunica vaginalis	«+»	4	4	4	4	4
	«–»	9	9	9	9	9
	p	0,04	>0,05	>0,05	>0,05	0,04
Invasion of spermatic cord	«+»	3	3	3	3	3
	«–»	10	10	10	10	10
	p	>0,05	>0,05	>0,05	>0,05	0,04

Summary

1. In the seminoma during transition from the initial to the late stages of tumor progression a significant increasing of S of CD31 and CD34 expression as well as VD is observed.

2. Indicators of CD31, CD34 expression and VD may be independent factors of prognosis of metastasis and seminoma progression.

3. Formation of intratumoral vessels in the seminoma occurs by angiogenesis and vasculogenesis with participation of progenitor EC.

4. Seminoma is characterized by vasculogenic mimicry in the form of channels formation that do not have endothelial lining.

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