Potapov Serhii M., Halata Daria I., Pliten Oksana M., Halata Anton I. Peculiarities of angiogenesis in testicular embryonal carcinoma. Journal of Education, Health and Sport. 2019;9(10):299-310. eISSN 2391-8306. DOI http://dx.doi.org/10.5281/zenodo.3546217
http://ojs.ukw.edu.pl/index.php/johs/article/view/7625
https://apcz.umk.pl/czasopisma/index.php/JEHS/article/view/7625

The journal has had 7 points in Ministry of Science and Higher Education parametric evaluation. Part B item 1223 (26/01/2017).
1223 Journal of Education, Health and Sport eISSN 2391-8306 7

Received: 03.10.2019. Revised: 08.10.2019. Accepted: 30.10.2019.

PECULIARITIES OF ANGIOGENESIS IN TESTICULAR EMBRYONAL CARCINOMA

Serhii M. Potapov[1], Daria I. Halata[1], Oksana M. Pliten[1], Anton I. Halata[2]

[1]Kharkiv National Medical University, Department of Pathological Anatomy,
Kharkiv, 61022, Ukraine
[2]Kharkiv State Medical Hospital of Emergency Care, Burn department,
Kharkiv, 61000, Ukraine

Abstract

Despite the relatively low incidence of testicular germ cell tumors (TGCT), which amount only 1% of all cancers in men worldwide in the epidemiological aspect, unlike tumors of other localizations, it does not become more frequent with age, but reaches its peak in young men of working age what attaches to the problem not only great medical, but also social significance.

Estimation of angiogenesis in the tumor is considered as one of the markers for predicting the course of disease, presence of metastases and sensitivity to antitumor antiangiogenic therapy. 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 endothelial cells (CD31 and CD34) for assessing the aggressiveness and prognosis of TGCT and, in particular, EC, are absent in the available literature sources.

Investigation of peculiarities of neoangiogenesis in the dynamics of testicular embryonal carcinoma 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 embryonal carcinoma occurs by angiogenesis and vasculogenesis with participation of progenitor endothelial cells; embryonal carcinoma is characterized by vasculogenic mimicry in the form of channels formation that do not have endothelial lining.

Key words*:* testicular germ cell tumors; embryonal carcinoma; angiogenesis; immunohistochemical investigation.

Despite the relatively low incidence of testicular germ cell tumors (TGCT), which amount only 1% of all cancers in men worldwide [1] in the epidemiological aspect, unlike tumors of other localizations, it does not become more frequent with age, but reaches its peak in young men of working age what attaches to the problem not only great medical, but also social significance [2, 3].

TGCT constitute more than 90% of all testicular tumors [1, 4]. Embryonal carcinoma (EC) belongs to non-seminomatous TGCT. Although it occurs as a «pure» tumor in only 2,3–16%, as a whole account for 40% of all TGCT and 87% of non- seminomatous tumors [1, 5].

The immunohistochemical (IHC) analysis of tumors uses a significant range of molecular biological markers. At that, the most important requirement for prognostic markers is their clear biological role, which is closely related to the aggressiveness of each single tumor [6].

Estimation of angiogenesis in the tumor is considered as one of the markers for predicting the course of disease, presence of metastases and sensitivity to antitumor antiangiogenic therapy [7-9].

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 [9].

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 endothelial cells (CD31 and CD34) for assessing the aggressiveness and prognosis of TGCT and, in particular, EC, are absent in the available literature sources.

Objectives: to study peculiarities of neoangiogenesis in the EC depending on stage of tumorous 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 testicular EC (including 7 cases when EC was a component of mixed TGCT) 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 [1], what is highly important, as the precise diagnosis and staging, which is made in compliance with the advanced science, are fundamental [10].

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 «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 (Ukrainian invention patent № 119922) [11].

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 [12, 13] 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 [12].

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

Table 1

Indicators of vascularization in the dynamics of testicular EC progression

	Group «1» (n_1=5)	Group «2» (n_2=4)	Group «4» (n_4=4)	p
CD34, S, %	2,46±0,77	2,09±0,06	2,48±0,08	p_{12}>0,05 p_{14}>0,05 p_{24}=0,021
CD34, L, unit.	39,0±0,19	40,29±0,16	35,94±2,56	p_{12}=0,014 p_{14}>0,05 p_{24}>0,05
CD31, S, %	1,96±0,56	2,03±0,09	2,79±0,08	p_{12}>0,05 p_{14}=0,035 p_{24}=0,021
CD31, L, unit.	34,82±1,37	39,06±0,15	39,51±0,21	p_{12}>0,05 p_{14}>0,05 p_{24}>0,05
VD, number of vessels in SFV	51,02±7,51	74,64±1,06	89,98±2,18	p_{12}>0,05 p_{14}=0,014 p_{24}=0,021

The analysis of CD34 showed that in the EC 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. In group «1», the average S of the CD34-positive staining was insignificant, and L corresponded to strong level. At the same time in one of five observations S of staining (5,50±0,21%) was significantly higher than that in each case ($p<0,05$) and in comparison with group indicator on average. In the tumor tissue small and medium caliber vessels prevailed, in which EC with membrane and cytoplasmic staining were well visualized.

Formation of new vessels in the EC occurred by angio- and vasculogenesis. The process of angiogenesis demonstrated formation of new vessels from previously existing

ones. Numerous linear formations branched from them still have no lumens or had appearance of tubular structures with unevenly widened lumens lined by endothelial cells. Here and there the newly formed vessels formed thick network around the epithelial complexes already at this stage of tumor progression. Vasculogenesis occurred at the expense of presence of a certain number of progenitor endothelial cells that were observed in the stromal component of tumors.

As for characteristics of tumor vessels in EC it should be noted that clear gradation of vessels by type (capillaries, arterioles, venules) was not observed. Instead, a chaotic network of vessels of various types was formed. Also so-called «sprouting» (excessive branching), curvature and other deformations which indicates immaturity were characteristic for microvessels. Immaturity of the vessels was also proved by frequent absence of pericytes in the intratumoral vessels and indistinctness of the basement membrane.

Evident intensification of neoangiogenesis was observed in areas of tumor invasion (Fig. 1, A), as well as near foci of necrosis and hemorrhages, which were quite frequent in EC. This phenomenon can be explained by the fact that development of necrosis results in release of biologically active pro-angiogenic substances that have an influence on vascular growth [14].

Besides, foci of necrosis attract macrophages, lymphocytes, platelets, mast cells which take participation in inflammatory reactions [15] and which may have a mitogenic effect on endothelial cells.

In group «2» the average S of CD34-positive staining did not differ from similar indicator in previous group and L of expression was on the boundary between strong and moderate level.

As well as in group «1» distribution of vessels in the tumor tissue visually looked non-uniformly and was characterized by the presence of a chaotic network of vessels of various types, often with signs of immaturity. At that, vessels of all indicated types were visualized in the stromal component of the tumors and in the glandular – mainly vessels of the capillary type. Visually, VD in the stromal component exceeded that in the glandular one (Fig. 1, B).

It should be noted that formation of new vessels in the glandular component occurred mainly by way of vasculogenesis, while in the stromal component through angio- and vasculogenesis. In this group, accumulations of progenitor endothelial cells were identified less frequent, what indicates the prevalence of angiogenesis.

In the observations of group «4» the average S of CD34 expression was significantly higher than that in group «2» and did not differ from the same indicator of group «1». L of CD34 expression was strong. Visually distribution of blood vessels, as well as the processes of angio- and vasculogenesis coincided with those in the previous groups.

In group «1» the average S of CD31-positive staining was small and L corresponded to strong level. In one of observations (the same case where overexpression of CD34 was observed) the S of CD31 immunopositive staining (4,21±0,03%) was significantly higher than that in each single case ($p<0,05$). In the stromal and glandular components of tumors some differences in the distribution of the vessels and VD were noted. Thus, in the stromal component randomly situated vessels of medium and small caliber were determined. Significant predominance of capillaries with good visible endothelial cell was detected as well. In sections of the capillaries which passed along their luminal surface endothelial cells which had appearance of monolayer with clear membrane staining were visualized. Neogenesis of microvessels also occurred by way of branching – way of angiogenesis. In glandular epithelial component small-caliber vessels with a typical membrane and less often membrane-cytoplasmic staining of endotheliocytes were determined.

In group «2» the average S of CD31-positive staining was at the same level of group «1» and L of marker corresponded to strong what did not significantly differ from similar indicators in the previous group. Visually VD was slightly higher than in the previous group and at comparison of glandular and stromal components of the tumor VD prevailed in the last one. VD in the central parts of the glandular complexes looked smaller than in their peripheral areas.

In the observations of group «4» S of CD31 expression increased and was significantly higher than in groups «1» and «2». L of CD31 as well as distribution of vessels in the investigated tumors, practically coincided with those in group «2». Visual VD (at CD31 expression) increased in comparison with groups «1» and «2» and was a little bit higher in stromal component. In the central parts of the glandular complexes in EC visual VD was considerably smaller than that in their peripheral areas (at the border with the stromal component) where the microvessels formed a thick network (Fig. 1, C). In areas of tumor invasion there was an increasing neoangiogenesis with the formation of a large number of vessels mainly of capillary type (Fig. 1, D).

In addition to neovascularization we observed in EC the phenomenon of vasculogenic mimicry which, as the researchers suggest is a component of malignant tumor biology along with inactivation of apoptosis, induction of angiogenesis, genome instability, escape from immune surveillance and the ability to metastasize [16].

The determination of VD was very important in the study of the tumor vascular component since this parameter as a prognostic criterion fully meets this requirement for the following reasons. First of all angiogenesis is necessary for the transition from pre-invasive (carcinoma «in situ») to the invasive stage of the primary tumor [17].

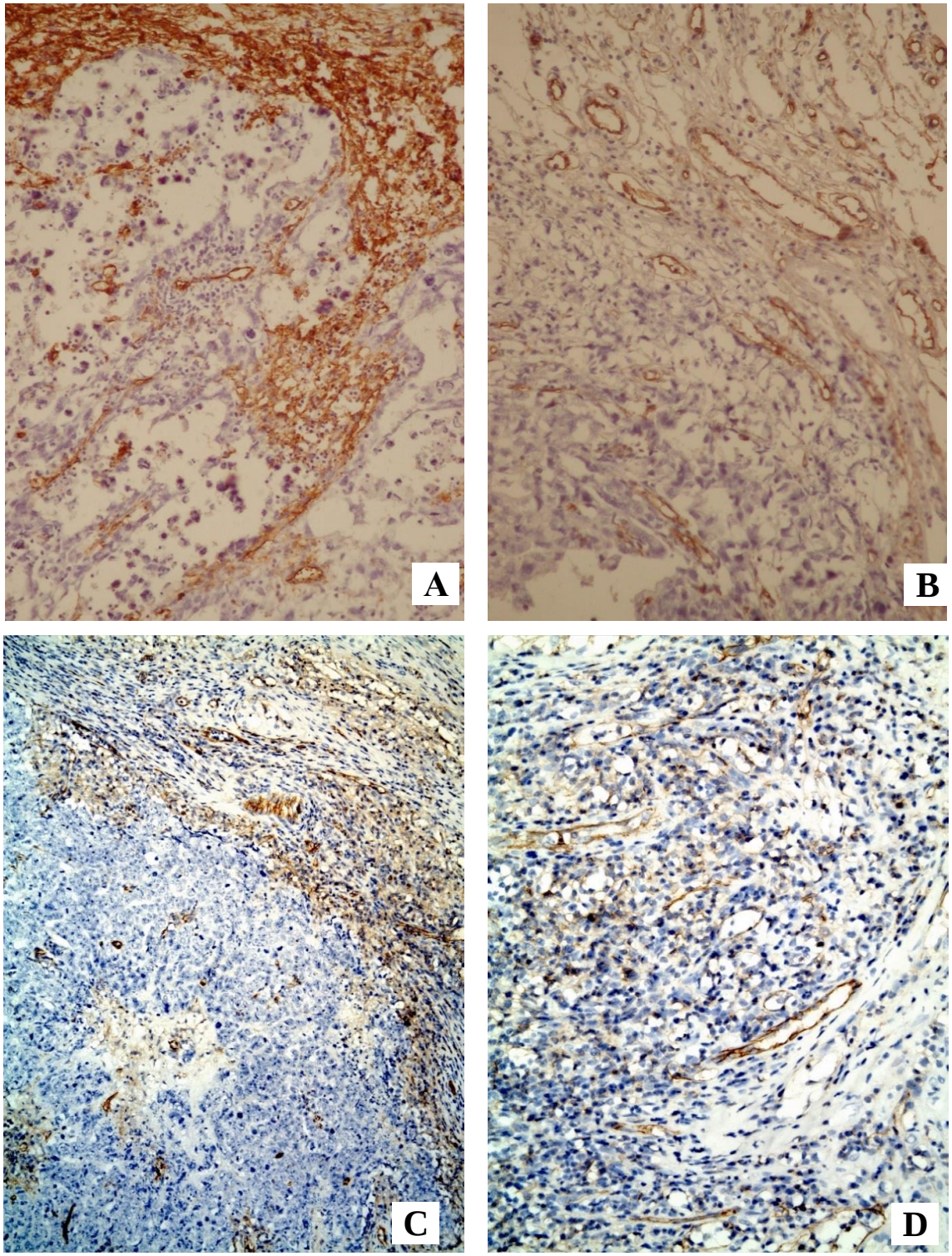

Fig. 1. A. Expressed neogenesis of the vessels with intensive CD34-positive staining in the area of invasion in EC of group «1» (×200). B. Prevalence of VD with moderate CD34-positive staining in the stromal component of EC of group «2» (×200). C. Intensive reaction with CD31 in the stromal component on the border with glandular structures of EC of group«4» (×100). D. Intensive reaction with CD31 in the developed network of newly formed capillaries in the area of invasion of EC of group «4» (×200). Immunoperoxidase reaction.

In the second place, in invasive tumors, even in the same histological types, the angiogenic activity of the tumor varies [18], what makes it possible to divide patients according to the degree of angiogenic activity of the tumor. And an increase of VD is considered as unfavorable factor in many tumors [19, 20].

A morphometric study showed that in EC of group «4» the average VD in the SFV was significantly higher than the same indicator in groups «1» and «2», which did not have significant differences between each other, what is explained by presence in the group «1» previously described observation with high S of CD34 expression and VD (80,65±0,80 number of vessels in SFV) in comparison with other cases in this group.

Thus, we can conclude that, in general, vascularization of EC was more significant in the late stages of tumor progression, which were characterized by the development of lympho- and hematogenous metastases.

Analysis of quantitative parameters of the investigated markers using nonparametric Spearman correlation coefficient found that, as the tumor progression stage increased, very high positive associations were observed between S of CD31 and CD34 expression with VD ($r= +0,92$; $r= +0,93$; $p<0,05$, respectively). At the same time, a very high positive association was established between S of CD31 expression and S of CD34 expression ($r= +0,96$; $p<0,05$).

So, the correlation analysis in combination with the mentioned above data demonstrated an increase of neoangiogenesis in the EC at tumor progression and also confirmed the effectiveness of the applied method for calculating of S of CD31 and CD34 expression.

It was also found that in patients with distant metastases S of CD31 and CD34 expression as well as VD were significantly higher than in patients without metastases. Besides that, in patients with vascular invasion VD was higher than in patients without it (table 2).

Table 2

Association of IHC parameters with EC aggressiveness

Mann-Whitney test						
		CD31, S, %	CD31, L, unit.	CD34, S, %	CD34, L, unit.	VD, number of vessels in SFV
Distant metastases	«+»	4	4	4	4	4
	«–»	9	9	9	9	9
	p	0,03	>0,05	0,03	>0,05	0,01
Vascular invasion	«+»	8	8	8	8	8
	«–»	5	5	5	5	5
	p	>0,05	>0,05	>0,05	>0,05	0,02

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

Summary

1. In the EC 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 EC progression.

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

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

References:

1. Moch H, Humphrey PA, Ulbright TM, Reuter VE, editors. WHO Classification of Tumours of the Urinary System and Male Genital Organs (4th edition). *Lyon: IARC*; 2016. 356 p.

2. Loran OB, Bogdanov AB, Ivkin EV, Sokolov EA, Metelyov AYu. Radikalnyye i organosokhranyayushchiye operatsii pri zlokachestvennykh novoobrazovaniyakh yaichka [Radical and testicular-sparing surgery for testicular malignancy]. *Russian Journal of Surgery*. 2014;1:25-30. (in Russian)

3. Nosov AK, Mamizhev EM, Vorobev AV, Zhukov OB, Novikov AI, Zassev RD, Korneev IA, Petrov SB. [Incidentalomas of the testicle and testicular microlithiasis: Current approaches to diagnosis and treatment (literature review, clinical cases)]. *Andrology and Genital Surgery*. 2017;1:28-38. (in Russian)

4. Vozianov OF, Romanenko AM, Klymenko IO. Onkourolohiia sohodni: dosiahnennia, problemy, perspektyvy [Modern oncourology: achievements, problems, and outlooks]. *Onkolohyia*, 2006;2:152–158. (in Ukrainian)

5. Andreyeva Yu.Yu, Frank GA, editors. Opukholi yaichka. Morfologicheskaya diagnostika i genetika: Rukovodstvo dlya vrachey. *Moskva: RMAPO*; 2011. 54 s. (in Russian)

6. McGuire WL. Guidelines for evaluating new prognostic factors. *J.Nat. Cancer Inst*., 1991;83:154–155.

7. Babichenko II, Kovyazin VA. Novyye metody immunogistokhimicheskoy diagnostiki opukholevogo rosta: Uchebnoe posobiye. *Moskva: RUDN*; 2008. 109 s. (in Russian)

8. Khlebnikova AN, Novoselova NV. Osobennosti angiogeneza v ochagakh bazalno-kletochnogo raka kozhi [Particular features of angiogenesis in lesions in patients suffering from basal cell epithelioma]. *Vestnik dermatologii i venerologii*, 2014;3:60–64. (in Russian)

9. Nefedova NA, Kharlova OA, Danilova NV, Malkov PG, Gayfullin NM. Markery angiogeneza pri opukholevom roste [Markers of angiogenesis in tumor growth]. *Arkhiv patologii*, 2016;2:55–62. (in Russian)

10. Lobo J, Costa AL, Vilela-Salgueiro B, Rodrigues Â, Guimarães R, Cantante M,Lopes P, Antunes L, Jerónimo C, Henrique R. Testicular germ cell tumors: revisiting a series in light of the new WHO classification and AJCC staging systems, focusing on challenges for pathologists. *Hum Pathol*. 2018 Dec; 82:113-124. doi: 10.1016/j.humpath.2018.07.016.

11. Potapov SM, Markovskiy VD, KulIshova NE, vynakhidnyky; Kharkivskyi natsionalnyi medychnyi universytet, patentovlasnyk. Sposib kilkisnoi otsinky rivnia svitlosti ta vidnosnoi ploshchi ekspresii markeriv pry imunohistokhimichnomu doslidzhenni tkanyn. Patent Ukrainy №119922. 2019 Serp 27. (in Ukrainian).

12. Kobzar AI. Prikladnaya matematicheskaya statistika. Dlya inzhenerov i nauchnyih rabotnikov. *Moskva: Fizmatlit*; 2012. 816 s. (in Russian)

13. Runyon RP. Nonparametric Statistics: A Contemporary Approach (Addison-Wesley series in statistics). Addison-Wesley Publishing Co; 1977. 218 p.

14. Kurtukova MO, Bugayeva IO, Ivanov AN. Faktory reguliruyushchiye angiogenez [Factors regulating angiogenesis]. *Sovremennyye problemy nauki i obrazovaniya*, 2015;5. (in Russian)

15. Kushlinskiy DN, Tereshkina IV, Degtyar VG, Laktionov KP, Adamyan LV. Faktor rosta endoteliya sosudov i ego retseptory pri rake yaichnikov [Endothelial growth factor and its receptors in ovarian cancer] / D.N. Kushlinskiy. *Molekulyarnaya meditsina*, 2013;1:3–11. (in Russian)

16. Vartanyan AA. Molekulyarnyye mekhanizmy vaskulogennoy mimikrii pri zlokachestvennykh zabolevaniyakh [dysertatsiia]. Moskva: Rossiyskiy onkologicheskiy nauchnyy tsentr imeni H.H. Blokhina; 2012. 39 s. (in Russian)

17. Guidi AJ, Fischer L, Harris JR, Schnitt SJ. Microvessel density and distribution in ductal carcinoma in situ of the breast. *J Natl Cancer Inst*., 1994 Apr 20;86(8):614-9.

18. Gasparini G, Weidner N, Bevilacqua P, Maluta S, Dalla Palma P, Caffo O, Barbareschi M, Boracchi P, Marubini E, Pozza F. Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. *J Clin Oncol*., 1994 Mar;12(3):454-66.

19. Lee SK, Cho EY, Kim WW, Kim SH, Hur SM, Kim S, Choe JH, Kim JH, Kim JS, Lee JE, Nam SJ, Yang JH. The prediction of lymph node metastasis in ductal carcinoma in situ with microinvasion by assessing lymphangiogenesis. *J Surg Oncol*., 2010 Sep 1;102(3):225-9. doi: 10.1002/jso.21607.

20. Nico B, Benagiano V, Mangieri D, Maruotti N, Vacca A, Ribatti D. Evaluation of microvascular density in tumors: pro and contra. *Histol Histopathol*., 2008 May;23(5):601-7. doi: 10.14670/HH-23.601.