al growth factor-A (VEGF-A) binding to neuropilin-1. J Biol Chem. 2012 Mar 30;287(14):11082-9. doi: 10.1074/jbc.M111.331140.

29. Pollock CE, Sutherland HG, Maher BH, et al. The NRP1 migraine risk variant shows evidence of association with menstrual migraine. J Headache Pain. 2018 Apr 18;19(1):31. doi: 10.1186/s10194-018-0857-z.

30. Pellet-Many C, Frankel P, et al. Neuropilins: structure, function and role in disease. Biochem J. 2008 Apr 15;411(2):211-26. doi: 10.1042/BJ20071639.

31. Siri M, Dastghaib S, Zamani M, et al. Autophagy, unfolded protein response, and neuropilin-1 cross-talk in SARS-CoV-2 infection: what can be learned from other coronaviruses. Int J Mol Sci. 2021 Jun 1;22(11):5992. doi: 10.3390/ijms22115992.

32. Sun B, Wu XY, Nie M. [SARS-CoV-2 damages male fertility: How and why]. Zhonghua Nan Ke Xue. 2021 Aug;27(8):738-741. Chinese. PMID: 34914248.

33. Vander Kooi CW, Jusino MA, Perman B, et al. Structural basis for ligand and heparin binding to neuropilin B domains. Proc Natl Acad Sci U S A. 2007 Apr 10;104(15):6152-7. doi: 10.1073/pnas.0700043104.

34. Xu J, Qi L, Chi X, et al. Orchitis: a complication of severe acute respiratory syndrome (SARS). Biol Reprod. 2006 Feb;74(2):410-6. doi: 10.1095/biolreprod.105.044776.

35. Zhao Y, Zhu R, Xiao T, Liu X. Genetic variants in migraine: a field synopsis and systematic re-analysis of meta-analyses. J Headache Pain. 2020 Feb 11;21(1):13. doi: 10.1186/s10194-020-01087-5.

POINT OF VIEW

I.V. Kononova, S.N. Mamaeva, V.A. Alekseev

DETECTION OF BETA-GLOBIN GENES IN THE ERYTHROCYTE FRACTION IN A PATIENT WITH CERVICAL CANCER

DOI 10.25789/YMJ.2022.79.22

УДК 611.018.26:616-056.52

The results of detection of DNA fragments encoding human beta-globin and human papillomavirus (HPV) L1 protein in cervical cancer patient's plasma and erythrocyte samples using real-time polymerase chain reaction (qPCR) are presented. Amplification of a DNA fragment encoding human beta-globin was found only in a sample of erythrocytes, which may indirectly indicate the presence of extracellular DNA. The results of qPCR for the presence of a DNA fragment encoding HPV L1 were negative, which did not allow us to confirm that the isolated DNA belonged to the tumor DNA. The origin of the identified in the erythrocyte fraction DNA fragment encoding human beta-globin requires further research.

Keywords: tumor DNA, extracellular vesicles, neutrophil extracellular traps.

Beta-globin is a part of hemoglobin - an iron-containing metalloprotein. In mammals hemoglobin is found in the largest amount in red blood cells, its main function is to transport oxygen from the lungs to various body tissues. Hemoglobin also interacts with other gases such as carbon dioxide, carbon monoxide, and nitric oxide. Hemoglobin is a tetramer composed of two beta-globin chains and also of two alpha-globin chains. Heme is attached to each of these chains, and thus each chain can transport oxygen. Hemoglobin is expressed by erythroid cells as well as by nonerythroid cells, such as epithelial cells, including epithelial cells of the cervix uteri [22].

It has been established that in normal cells of the cervical and vaginal mucosa alpha-globin and beta-globin can act as endogenous antimicrobial protective proteins against infection [21]. Hemoglobin and mRNA are also found in human cervical carcinoma cell lines (SiHa and CaSki), and the expression of alpha-globin and beta-globin genes in them is significantly higher than in normal cells of the cervix uteri [14]. Expression of these genes improves the viability of tumor cells by suppressing oxidative stress [28].

Alpha-globin and beta-globin are encoded by genes located on chromosomes 16 and 11, respectively. The beta-globin gene cluster is packed into inactive heterochromatin in non-erythroid cells, while the alpha-globin genes are built into open chromatin conformations in all cell types [9]. Transcriptionally inactive heterochromatin plays a vital role in maintaining a stable structure of specialized chromosomal regions with repetitive DNA; loss of integrity in these regions of chromosomes can contribute to the cancer development [7].

DNA, including genomic DNA, is found in blood fractions purified from cells - in plasma and serum. Both normal and tumor cells release DNA into the circulation, but it is present in increased amounts in cancer patients [23]. It is assumed that the detection of tumor DNA in the blood can significantly improve the detection of a tumor at an early stage, determine its progression and prognosis, and also help in targeted therapy [5]. Detection of tumor DNA is carried out mainly in blood plasma, but the complexity of the determination is associated with low concentration of DNA in plasma [11].

Human genomic DNA is also found in the erythrocyte fraction of blood, even after 25 years of storage [4]. Genomic DNA, both of human and infectious, can be associated with the surface of erythrocytes through receptors, since it is known that erythrocytes express receptors on their surface, which, at least bind bacterial and mitochondrial DNA in vitro [13].

We assumed that in cervical cancer (CC) patient genomic DNA is contained in the blood in an increased amount, and the presence of beta-globin genes in genomic DNA can be an indicator of carcinogenesis, so the goal of the study was to isolate DNA from the patient's blood fractions in which there are no nuclear cells - from plasma and erythrocyte fraction, as well as to detect a DNA fragment encoding human beta-globin. In order to determine, let indirectly, whether the isolated DNA belongs to a tumor DNA, it was decided to detect a DNA fragment encoding the L1 protein region of the human papillomavirus (HPV). Since it is believed, that in CC patient circulating extracellular DNA containing the HPV genome originates from tumor cells [8].

Materials and methods. 4 types of biological samples were prepared from venous blood taken by venipuncture in vacuum blood collection tubes-K3 EDTA: plasma samples (sample 1); a suspen-

KONONOVA Irina Vasilyevna – PhD in Medicine, associate researcher, acting director, Arctic Medical Center, YSC of complex medical problems, irinakon.07@mail.ru, SPIN code: 3282-7170, ORCID: 0000-0002-9243-6623; **MAMAYEVA Sargylana Nikolaevna** – Ph.D. in Physics and Mathematics, head of the Department of the Institute of Physics and Technology, M.K. Ammosov NEFU, sargylana_mamaeva@mail.ru; **ALEKSEEV Vladislav Amirovich** – junior researcher Arctic Medical Center, YCS CMP.

The main results of the pilot study

	Sample 1 (plasma)	Sample 2 (erythrocyte suspension)	Sample 3 (kontrol - erythrocyte suspension, trypsin-treated)	Sample 4 (phosphate buffer precipitate)
DNA Isolation	(+)	(+)	(+)	(-)
For DNA fragment encoding human beta-globin:				
Amplification of a gene fragment encoding human beta-globin using qPCR	(-)	(+)	(-)	Not carried out
Verification of amplification by gel electrophoresis	(-)	(+)	(-)	Not carried out
Conclusion	**DNA fragment encoding human beta-globin found in erythrocyte suspension**			
For DNA fragment encoding human papillomavirus protein L1:				
Amplification of a gene fragment encoding human papillomavirus protein L1 using qPCR	(+/-) questionable	(-)	(-)	Not carried out
Verification of amplification by gel electrophoresis	(-)	(-)	(-)	Not carried out
Conclusion	**DNA fragment encoding HPV L1 protein was not found in any of the samples**			

sion of erythrocytes washed in phosphate buffer (sample 2); a suspension of trypsin-treated and then washed also in phosphate buffer erythrocytes (sample 3). The sample 3 served as a control for the absence of admixture of cells containing nuclei in the suspension of erythrocytes. In addition, the treatment of erythrocytes with trypsin was necessary to confirm the presence of the detected DNA fragments on the surface of erythrocytes, as much as it is known, that trypsin destroys cell surface receptors by proteolysis [10]. The phosphate buffer precipitate (sample 4), which was used to wash trypsin-treated erythrocytes, was also used for DNA detection.

The blood was centrifuged at 1600 rpm for 10 minutes to separate into fractions. Then, after collection and removal of the mononuclear cell plasma samples and erythrocyte fraction were isolated. The erythrocyte fraction was washed three times with phosphate buffer to obtain an erythrocyte suspension. In half of the erythrocyte suspension trypsin - 0.25% in a ratio of 1:1was added, and incubated at a temperature of 37°C for 10 minutes, after incubation was centrifuged for obtain a precipitation of erythrocytes; then the erythrocytes were washed three times with phosphate buffer.

All samples were stored in a freezer at –20°C before DNA extraction and real-time PCR.

DNA was isolated by phenol-chloroform extraction. The concentration and quality of the isolated DNA were determined using NanoPhotometer Pearl (Implen).

Real-time PCR (qPCR) was carried out on CFX96 Touch Real-Time PCR Detection System using qPCRmix-HS SYBR+LowROX reaction mixture (Evrogen).

The primers PC03/04 (5'-ACACAACTGTGTTCACTAGC-3'/5'-CAACTTCATCCACGTTCACC-3') were used to detect DNA fragments encoding human beta globin.

Primers MY09/11 (5'-CGTCCMARRGGAWACTGATC-3'/5'-GCMCAGGGWCATAAYAATGG-3') were used to detect DNA fragments encoding the HPV L1 protein.

The length of the amplicons was determined in an agarose gel.

The study was approved by the Local Committee on Biomedical Ethics of the North-Eastern Federal University named after M.K. Ammosov (Yakutsk, Sakha (Yakutia) Republic, Russia) in accordance with protocol No. 13 dated April 4, 2018, Decision No. 2. Patient B., a resident of Yakutia, with newly diagnosed cervical cancer, gave written informed consent to the study.

Results and discussion. The main results are presented in Table 1.

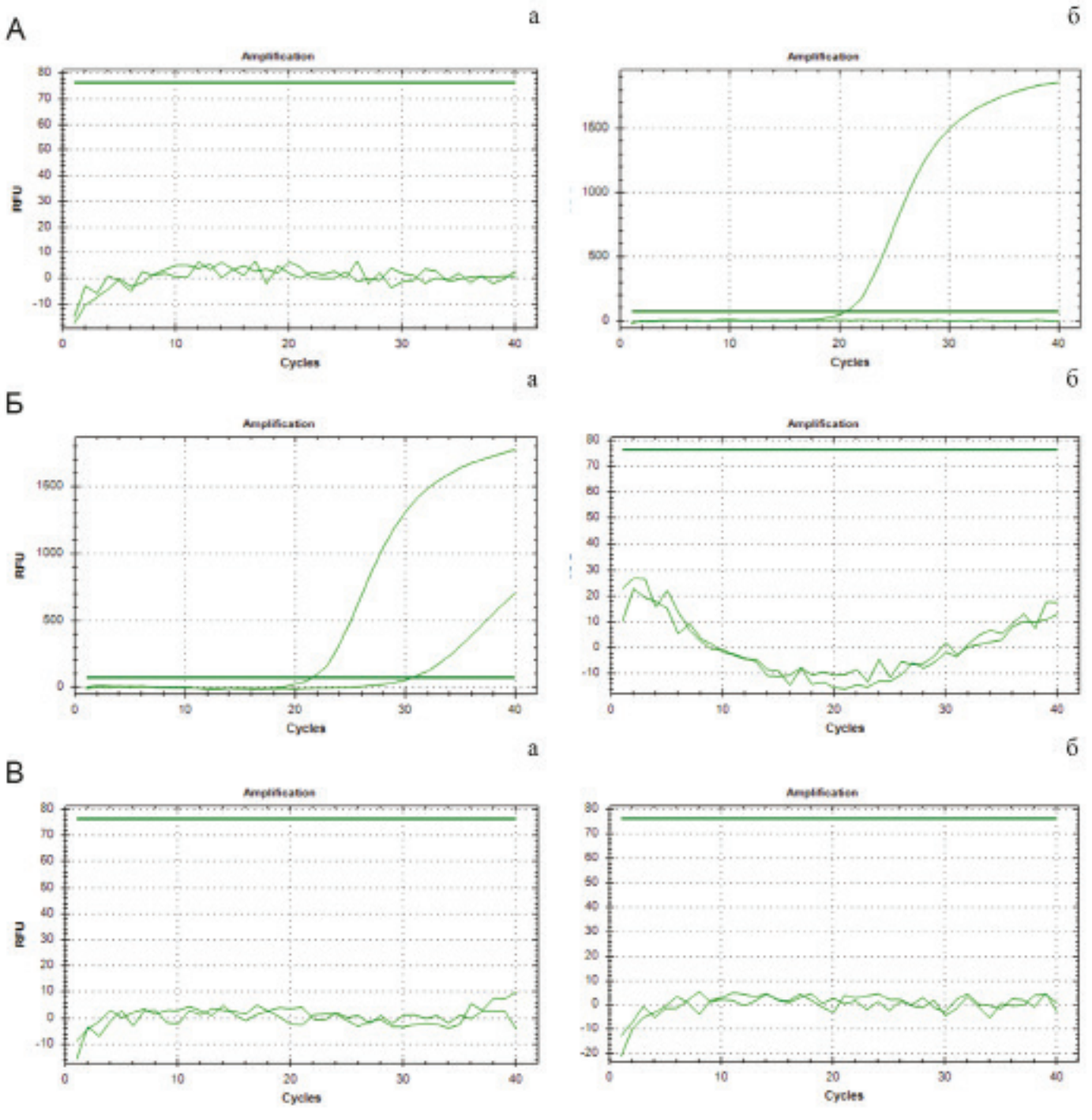
DNA was isolated from three samples - from plasma, a suspension of washed erythrocytes and erythrocytes treated with trypsin. DNA concentration in all of these samples exceeded the upper sensitivity limit of the spectrophotometer - 18750 ng/μl. In the fourth sample, a precipitate of phosphate buffer, which was used to wash erythrocytes treated with trypsin, DNA could not be isolated.

Sample 1 study. qPCR using primers PC03/04 and MY09/11 for DNA isolated from a plasma sample showed the absence of a fragment encoding human beta-globin, and a questionable result for a fragment encoding HPV L1 protein (Fig. 1). The electrophoresis confirmed the absence of amplification products of the DNA fragment encoding human beta-globin, and also showed the absence of amplification products of the DNA fragment encoding HPV L1 protein.

Sample 2 study. qPCR with DNA isolated from a sample of erythrocyte suspension revealed the presence of a fragment encoding human beta-globin, and the absence of a fragment encoding HPV L1 protein (Fig. 2). Electrophoresis confirmed the presence of amplification products for the DNA fragment encoding human beta-globin and the absence of amplification products for the DNA fragment encoding HPV L1 protein.

Sample 3 study. As for the DNA isolated from a suspension of erythrocytes treated with trypsin, using real-time PCR no detections of fragments encoding human beta-globin and encoding HPV L1 protein were (Fig. 3). Gel electrophoresis showed consistency with these results - showed the absence of amplification products for both DNA fragments.

Thus, in this case, DNA was found in blood fractions in which there are no cells containing nuclei - in plasma and erythrocytes. DNA containing a fragment encoding human beta-globin, was found only in the erythrocyte fraction of blood. The presence of this DNA in the erythrocyte fraction cannot be explained by poor-quality isolation of erythrocytes, that is, the presence of cells containing nuclei - reticulocytes and leukocytes in the erythrocyte fraction. Since the detection of DNA containing a fragment encoding human beta-globin did not occur after treating erythrocytes with trypsin. This also suggests that, most likely, this DNA is associated with the surface of erythrocytes through receptors, since trypsin is able to destroy by proteolysis receptor bonds. The absence of DNA fragments encoding HPV L1 in the erythrocyte fraction reduces the likelihood of DNA origi-



The fluorescent signal accumulation of the amplification of DNA fragments isolated from plasma: a - amplification of a DNA fragment encoding human beta-globin, b - encoding HPV L1 protein

nating from cervical tumor cells, although, of course, this statement is not obvious. It is possible that the isolated DNA belongs to neutrophil extracellular traps (neutrophil extracellular trap, NET), which play a unique role in carcinogenesis [17, 25]. And, perhaps, exactly neutrophil extracellular traps can be determined in the erythrocyte fraction earlier than in plasma and serum. Currently, researchers have shown that plasma and serum levels of neutrophil extracellular traps correlate with the progression of cancer - colorectal [26], breast [20], and stomach [27]. Endogenous extracellular particles that were visualized on the surface of erythrocytes [15], including those in CC patients [16], can also be a possible source of isolating DNA containing a fragment encoding human beta-globin. For example, it has been established in pancreatic cancer patients that endogenous extracellular particles, such as, serum exosomes, contain human genomic DNA [6].

The question is the origin of DNA, which does not contain the sought for fragments, isolated from plasma and erythrocyte suspension treated with trypsin. It is not excluded that DNA isolated from plasma may be of infectious origin. Since CC patients often have positive molecular genetic tests for the presence of other infections, for example, sexually transmitted ones [1, 2, 24]. In cervical samples, even with negative results of HPV DNA detection, the results of tests

for herpes virus DNA can be positive [3], and herpes virus DNA is also detected in plasma [12].

The infectious origin of DNA isolated from a suspension of erythrocytes treated with trypsin also is not excluded. Perhaps, its detection in this sample indicates the presence of DNA-containing immune complexes on the erythrocyte surface. It has been established that trypsin destroys not all receptor bonds, for example, surface cell receptors for IgG are resistant to its action [18], probably due to the fact that the proteolytic activity of trypsin is still specific [19].

Conclusion. In CC patient genomic DNA was isolated and detected in increased amounts in plasma and erythrocyte fraction, including after treating with trypsin. DNA containing a fragment encoding human beta-globin, in the absence of its detection in plasma, was detected in the erythrocyte fraction, probably in a bound state to the surface of erythrocytes by receptors. The question of whether the isolated DNA belongs to the tumor DNA is debatable, especially since the DNA fragment encoding HPV L1 protein was not found in any of the fractions. The origin of the isolated DNA should be studied in the future.

Reference

1. Abebe M., Eshetie S., Tessema B. Prevalence of sexually transmitted infections among cervical cancer suspected women at University of Gondar Comprehensive Specialized Hospital, North-west Ethiopia. BMC Infectious Diseases. 2021;21. doi: https://doi.org/10.1186/s12879-021-06074-y

2. Alotaibia H.J., Almajhdia F.N., Alsaleh A.N. [et al.]. Association of sexually transmitted infections and human papillomavirus co-infection with abnormal cervical cytology among women in Saudi Arabia. Saudi Journal of Biological Sciences. 2020;27(6):1587-1595. doi: https://doi.org/10.1016/j.sjbs.2020.03.021

3. Bahena-Román M., Sánchez-Alemán M.A., Contreras-Ochoa C.O. [et al.]. Prevalence of active infection by herpes simplex virus type 2 in patients with high-risk human papillomavirus infection: A cross-sectional study. Journal of Medical Virology. 2020; 92: 1246– 1252. https://doi.org/10.1002/jmv.25668

4. Buchanan A.V., Sherry S.T., Weiss K.M. [et al.]. Extraction of DNA from Frozen Red Blood Cells. Human Biology. 199;65(4):647–654. doi: http://www.jstor.org/stable/41465130

5. Cheng F., Su L., Qian C. Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer. Oncotarget. 2016;7(30):48832-48841. doi: 10.18632/oncotarget.9453

6. DNA in extracellular vesicles: from evolution to its current application in health and disease / J. Ghanam [et al.] // Cell and Bioscience. - 2022. - № 12. - Article number: 37. - https://doi.org/10.1186/s13578-022-00771-0

7. Duggan N. M., Tang Z. I. The Formation of Heterochromatin. Nature Education. 2010;3(9). URL: https://www.nature.com/scitable/topicpage/the-formation-of-heterochromatin-and-rna-interference-14169031/ (Date of access July,18, 2022)

8. Gu Y., Wan c., Qiu J. Circulating HPV cDNA in the blood as a reliable biomarker for cervical cancer: A meta-analysis. PLoS One. 2020;15(2). doi: 10.1371/journal.pone.0224001

9. Hardison R. Hemoglobins from bacteria to man: evolution of different patterns of gene expression. Journal of Experimental Biology. 1998;201(Pt 8):1099-1117. doi: 10.1242/jeb.201.8.1099

10. Huang H.L., Hsing H.W., Lai T.C. [et al.]. Trypsin-induced proteome alteration during cell subculture in mammalian cells..Journal of Biomedical Science. 2010;17. doi: https://doi.org/10.1186/1423-0127-17-36

11. Jin C.E., Koo B., Lee T.Y. [et al.]. Simple and Low-Cost Sampling of Cell-Free Nucleic Acids from Blood Plasma for Rapid and Sensitive Detection of Circulating Tumor DNA. Advanced Science. 2018; 5. doi: https://doi.org/10.1002/advs.201800614

12. Juhl D. Detection of herpes simplex virus DNA in plasma of patients with primary but not with recurrent infection: implications for transfusion medicine? Transfusion Medicine. 2010;20:38-47. doi: https://doi.org/10.1111/j.1365-3148.2009.00951.x

13. Lam L.K.M., Murphy S., Venosa A. [et al.]. DNA binding to TLR9 expressed by red blood cells promotes innate immune activation and anemia. Science Translational Medicine. 2021; 13(616). doi: 10.1126/scitranslmed.abj1008

14. Li X., Wu Z., Wang Y. [et al.]. Characterization of adult α- and β-globin elevated by hydrogen peroxide in cervical cancer cells that play a cytoprotective role against oxidative insults. PLoS One. 2013;8(1). doi: 10.1371/journal.pone.0054342

15. Mamaeva S.N., Kononova I.V., Munkhalova Ya.A. [et al.]. Issledovanie eritrocitov pacientki s hronicheskim mezangioproliferativnym glomerulonefritom metodom skaniruyushchej elektronnoj mikroskopii [The Study of erythrocytes of a patient with chronic mesangial proliferative glomerulonephritis by scanning electron microscopy]. Sovremennie problemy nauki i obrazovania [Modern problems of science and education].2020;3:id=29910. doi: 10.17513/spno.29910. - URL: https://science-education.ru/ru/article/view?id=29910 (In Russ.)

16. Mamaeva S.N., Kononova I.V., Ruzhansky M. [et al.]. Using Scanning Electron Microscopy and Atomic Force Microscopy to Study the Formation of Nanoparticles on Red Blood Cell Surface in Cervical Cancer Patients. International Journal of Biomedicine. 2020;10(1):70-75. doi: 10.21103/Article10(1)_OA12

17. Masucci M.T., Minopoli M., Del Vecchio S., Carriero M.V. The Emerging Role of Neutrophil Extracellular Traps (NETs) in Tumor Progression and Metastasis. Frontier Immunology. 2020;11. doi: 10.3389/fimmu.2020.01749

18. Mingari M.C., Moretta L., Moretta A. [et al.]. Fc-receptors for IgG and IgM immunoglobulins on human T lymphocytes: mode of re-expression after proteolysis or interaction with immune complexes. Journal of Immunology. 1978;121(2):767-70

19. Olsen J.V., Mann M. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Technology. 2004;3(6):608-614. doi: https://doi.org/10.1074/mcp.T400003-MCP200

20. Rivera-Franco M.M., Leon-Rodriguez E., Torres-Ruiz J.J. [et al.]. Neutrophil extracellular traps associate with clinical stages in breast cancer. Pathology and Oncology Research. 2020;26(3):1781–1785. doi:https://doi.org/10.1007/s12253-019-00763-5

21. Saha D., Koli S., Patgaonka M. Expression of hemoglobin-α and β subunits in human vaginal epithelial cells and their functional significance. PLoS One. 2017;12(2). doi: 10.1371/journal.pone.0171084

22. Saha D., Patgaonkar M., Shroff A. Hemoglobin expression in nonerythroid cells: novel or ubiquitous? International Journal of Inflammation. 2014;2014:ID 803237. doi: 10.1155/2014/803237

23. Sorenson G.D., Pribish D.M., Valone F.H. [et al.]. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiology Biomarkers Prevention. 1994;(1):67-71

24. Tantengco O. A., Nakura Y., Yoshimur M. [et al.]. Co-infection of human papillomavirus and other sexually transmitted bacteria in cervical cancer patients in the Philippines. Gynecologic Oncology Report. 2022;40. doi: 10.1016/j.gore.2022.100943

25. Yang, L., Liu, Q., Zhang, X. [et al.] DNA of neutrophil extracellular traps promotes cancer metastasis via CCDC25. Nature. 2020;583:133–138. doi: https://doi.org/10.1038/s41586-020-2394-6

26. Yazdani H.O., Roy E., Comerci A.J. [et al.] Neutrophil extracellular traps drive mitochondrial homeostasis in tumors to augment growth. Cancer Research. 2019;79(21):5626–5639. doi: https://doi.org/10.1158/0008-5472.CAN-19-0800

27. Zhang Y., Hu Y., Ma C. [et al.] Diagnostic, therapeutic predictive, and prognostic value of neutrophil extracellular traps in patients with gastric adenocarcinoma. Frontier Oncology. 2020;10. doi: https://doi.org/10.3389/fonc.2020.01036

28. Zheng Y., Miyamoto D., Wittner B. [et al.]. Expression of β-globin by cancer cells promotes cell survival during blood-borne dissemination. Nature Communications. 2017; 8. doi: 10.1038/ncomms14344