Kabachenko V. O., Shishkin M. A. IHC characteristics of stem cell markers in PDAC with different degrees of differentiation. Journal of Education, Health and Sport. 2022;12(6):229-237. eISSN 2391-8306. DOI http://dx.doi.org/10.12775/JEHS.2022.12.06.023
https://apcz.umk.pl/JEHS/article/view/JEHS.2022.12.06.023
https://zenodo.org/record/6678066

The journal has had 40 points in Ministry of Education and Science of Poland parametric evaluation. Annex to the announcement of the Minister of Education and Science of December 1, 2021. No. 32343. Has a Journal's Unique Identifier: 201159. Scientific disciplines assigned: Physical Culture Sciences (Field of Medical sciences and health sciences); Health Sciences (Field of Medical Sciences and Health Sciences).

Punkty Ministerialne z 2019 - aktualny rok 40 punktów. Załącznik do komunikatu Ministra Edukacji i Nauki z dnia 1 grudnia 2021 r. Lp. 32343. Posiada Unikatowy Identyfikator Czasopisma: 201159. Przypisane dyscypliny naukowe:Nauki o kulturze fizycznej (Dziedzina nauk medycznych i nauk o zdrowiu); Nauki o zdrowiu (Dziedzina nauk medycznych i nauk o zdrowiu).

Received:11.05.2022. Revised: 31.05.2022. Accepted: 21.06.2022.

IHC CHARACTERISTICS OF STEM CELL MARKERS IN PDAC WITH DIFFERENT DEGREES OF DIFFERENTIATION

V. O. Kabachenko, M. A. Shishkin

Zaporizhzhia State Medical University

Zaporizhzhia, Ukraine

Shyshkin Maksym Andriiovych, MD, PhD, Associate Professor of the Department of Pathological Anatomy and Forensic Medicine, ZSMU; Maiakovskyi avenue, 26, Zaporizhzhia, 69035, Ukraine, shishkin.stomat@gmail.com; ORCID number 0000-0002-8979-8463

Kabachenko Valeriia Oleksandrivna, postgraduate PhD of the Department of Pathological Anatomy and Forensic Medicine, ZSMU; Maiakovskyi avenue, 26, Zaporizhzhia, 69035, Ukraine, leranaumenkokabachenko@gmail.com; ORCID 0000-0002-7219-6634

Abstract

Introduction. Cancer stem cells (CSCs) play a key role in the development, progression and metastasis of cancer. Cell surface markers, in particular CD44 and EpCAM, are used to identify CSCs. Marker expression patterns depend on the stage of progression and differentiation. **Materials and methods.** Histopathological and IHC studies of 49 cases of the pancreas ductal adenocarcinoma (PDAC) of G2, G3 degrees of differentiation. Results of IHC reactions with antibodies against CD44 and EpCAM were estimated by photo digital morphometry and were expressed in immunostained cells relative area (%). **Results.** Adenocarinoma of the pancreas is characterized by a moderate level of expression of cell surface markers CSC - CD44 and EpCAM. The expression level of CD44 in the I gr. - Me = 34.11% (Q1 = 31.03; Q3 = 38.39), in the II gr. - Me = 43.17% (Q1 = 39.83; Q3 = 47.04),

significantly higher than in the I gr. (Kruskal-Wallis test $p<0.05$). The expression level of EpCAM in the I gr. - Me=26,10% (Q1=20,42; Q3=31,89), in the II gr. - Me=31,07% (Q1=27,03; Q3=34,00) ($p>0,05$). In both groups, the relative area of EpCAM was significantly lower than CD44 (Kruskal-Wallis test $p<0.05$). Correlation analysis (Pearson's ratio) showed a weak negative relationship between markers at G2 ($r = -0.17$, $p>0.05$), at G3 - a weak positive ($r = + 0.06$, $p>0.05$).

Key words: Pancreas; Ductal adenocarcinoma; Cancer stem cells; EpCAM; CD44.

Introduction. A critical role in the occurrence, progression, and metastasis of cancer is assigned to cancer stem cells (CSC). A characteristic feature of pancreatic ductal adenocarcinoma (PDAC) as a malignant tumor is significant heterogeneity in subpopulations of these cells [1-5]. CSCs are exclusively tumor-generating and pluripotent; they are localized among cancer cells and have properties which are similar to stem cells. They range from 1% to 5% of the total number of tumor cells [1-5]. Research has also identified the characteristics of CSCs inherent in the epithelial-mesenchymal transition (EMT), which promotes the spread of cancer cells and metastasizes to other organs. In turn, it has been proven that the induction of EMT in cancer cells contributes to the appearance of symptoms similar to CSC [6-9]. During treatment, tumor cells dedifferentiate to stem cells and create a new pool of CSCs resistant to chemotherapy and radiation therapy, with unlimited self-healing and the ability to regenerate cell heterogeneity, which causes treatment failure and recurrence of PDAC [10, 11]. That is, CSCs have more pronounced invasiveness and metastasis in comparison with their differentiated analogues of cancer cells, innate higher chemo- and radioresistance. Unique cell surface markers in these cells that emphasize their heterogeneity are used to identify CSCs [12]. CD44 is a non-kinase receptor for transmembrane adhesion, a benign molecular marker of CSC. CD44 expression is associated with EMT regulation. Literature data indicate a correlation between overexpression of the CD44 marker and a low degree of differentiation, high proliferative activity and higher metastatic potential in the liver and lymph nodes [1-5, 13]. Scientific studies have established tumor recurrence after treatment due to CD44 + cells [2-5].

The epithelial cell adhesion molecule EpCAM, also known as epithelial specific marker (ESA), is used as another RSC biomarker. The effect of EpCAM on cell proliferation, partial association with EMT and, accordingly, a positive correlation with the progression of

PDAC, have been established [1-5]. However, in other scholarly sources, high expression of EpCAM is associated with a good prognosis [6-8].

CSCs differ in the number of differentiated cellular features even in one tumor, which may cause different patterns of expression of these markers at different stages of progression and differentiation [5-8]. In addition, CD44 and EpCAM are also expressed in normal stem cells [6-7]. Thus, the study of the expression of cell surface markers at different degrees of PDAC differentiation is necessary for early diagnosis and prevention of metastasis and recurrence.

The aim is to investigate the diagnostic significance of cell surface markers CD44 and EpCAM CSC at different degrees of PDAC differentiation to predict the course of the disease.

Materials and methods. A comprehensive pathomorphological, histochemical and immunohistochemical (IHC) study of the operative material of 49 PDAC cases has been performed. Patients were divided into two groups: group 1 comprised patients with moderate (G2) degree of differentiation (26 cases), group 2 included patients with low (G3) degree of tumor differentiation (23 cases). The age of patients ranged from 39 to 83 years, the average age was 62.15 ± 2.34 years. The control group included autopsy material of the pancreatic gland (PG) of 10 deceased patients aged 56-73 years without clinical and morphological data of pancreatic disease. Research material: pieces of 10% formalin-fixed paraffin-embedded pancreatic tissue. Paraffin-embedded serial sections (4 μm) were dewaxed according to the standard scheme and stained with hematoxylin and eosin to study the histological structure of PDAC. The histochemical staining method (Masson-trichrome staining) was used to study the microscopic features of the PDAC stroma.

Immunohistochemical (IHC) studies were performed on dewaxed and rehydrated serial sections (4 μm thick) according to standard methods, using primary antibodies against CD44 (Clone HCAM Ab-4) and epithelial specific antigen (EpCAM (Ab-9)). The sections were stained with Mayer's hematoxylin and embedded in the balm. The results of the IHC analysis were studied and evaluated using an Axioplan 2 microscope (Carl Zeiss, Germany), and sections were photographed (5 standardized microscopic fields of view (SMR), magnification x200) with an Axiocam 105 Color ZEISS digital camera (Germany). The expression levels of the studied IHC markers were assessed by photo-digital morphometry using the medical digital image processing software ImageJ, using the Color Deconvolution plug-in, and the color analysis scheme "HDAB" (hematoxylin + DAB) in automatic mode. To morphometrically measure the relative area of immunopositive cells (CD44 and EpCAM) in a

filtered DAB image channel, a standardized sensitivity threshold (Threshold tool) was set to segment the image; it divides all pixels into two types: white and black. The next step was to calculate the relative area occupied by immunopositive cells as a percentage of the number of pixels in the digital image of the positive IHC response to the total number of pixels in the image. The results were calibrated into three groups: low relative area of IHC-positive cells - 0-25%, moderate - from 26% to 75%, and high - from 76% to 100% of the relative area of immunopositive stained cells.

The results were statistically processed on a personal computer with "STATISTICA 13.0" (StatSoft Inc., license No. JPZ804I382130ARCN10-J) software. The median (Me), lower and upper quartiles (Q1; Q3) were calculated. Comparative analysis in the study groups was performed using the Kruskal-Wallis criteria. Pearson's coefficient was used for correlation analysis. The results were considered statistically significant at a value of $p < 0.05$.

Results. Analysis of CD44 expression in both study groups revealed in 100% of cases diffuse membrane-cytoplasmic staining in stromal cells, characterized by a small nucleus and a shift of the nuclear-cytoplasmic ratio towards the cytoplasm. With moderate differentiation, CD44 expression was observed around small tubular and trabecular complexes. Besides, in tumors with a moderate degree of differentiation, single foci of partial membrane expression of CD44 were observed by cancer-altered cells of tubules and large trabeculae. At a low degree of differentiation, diffuse brown membrane-cytoplasmic expression of CD44 was observed in large cancer-altered trabeculae. The relative area of expression of the stem cell marker in group 1 was Me = 34.11% (Q1 = 31.03; Q3 = 38.39). In group 2, the expression level of Me = 43.17% (Q1 = 39.83; Q3 = 47.04), significantly higher than in 1 (Kruskal-Wallis test $p < 0.05$). In tumors with a moderate degree of differentiation, the minimum expression was 24.56%, the maximum - 48.62%. In tumors with a low degree of differentiation, the minimum expression of CD44 was 1.57 (38.47%) times higher than in group 1, the maximum was 56.61%.

During the microscopic study of EpCAM expression, in contrast to CD44, no immunopositive EpCAM cells were detected in the stroma. In tumors with G2 degree of differentiation, diffuse brown membrane-cytoplasmic staining of EpCAM cancer-altered cells of small tubular complexes and large trabecular structures was observed in 100% of cases. The relative area of marker expression in this group was Me = 26.10% (Q1 = 20.42; Q3 = 31.89). At G3 degree of differentiation, diffuse brown membrane-cytoplasmic staining of immunopositive cells in similar but larger structures was also observed at 100%. In addition, the difference in EpCAM expression was secured by layers formed by individual small

immunopositive cancer cells and detected in the periphery. The relative area of marker expression in this group was equal to Me = 31.07% (Q1 = 27.03; Q3 = 34.00) without a significant difference from 1 gr. (p> 0.05). The minimum level of EpCAM expression in tumors with a moderate degree of differentiation was 7.88%, which is three times less than in tumors with a low degree of 22.28%. The maximum level of marker expression in group 1 was 40.54%, in group 2 - 39.06%.

In both groups, the relative area of EpCAM was significantly lower than CD44 (Kruskal-Wallis test, p <0.05).

Correlation analysis (Pearson's ratio) showed a weak negative relationship between markers at G2 (r = -0.17, p> 0.05), and a weak positive one at G3 (r = + 0.06, p> 0.05).

Discussion. Among all malignant processes, PDAC is aggressive [1-5]. The analysis is consistent with these data. Only moderate (G2 - 45.65%) and low (G3 - 54.35%) degrees of differentiation of PDAC with the predominance of a more aggressive form were found in the studied samples. It is believed that initiation and aggression are provoked by stem cells, which are a manifestation of the heterogeneity of malignant tumors, accounting for only 1-5% of all cancer cells [1-5]. Cell surface markers CD44 and EpCAM confirmed the presence of the corresponding cell subclone in PDAC.

The analysis noted not only an increase in CD44 expression in PDAC, but also a redistribution of protein from stromal cells, more pronounced in G3 degree. A similar feature of marker expression has been described in low-grade adenocarcinomas of the bladder [13]. According to the literature, with the help of some signaling pathways, stromal cells support and promote CSC populations [14, 15, 16]. In particular, it is believed that carcinogenic fibroblasts (CAF) can interact with a certain CD44 cell surface protein, causing a malignant phenotype or initiating a tumor [16, 17]. In addition, CD44-positive CAFs increase the survival and resistance of malignant cells, and inhibit apoptosis, as confirmed, for example, in breast cancer [14]. Thus, CD44 expressed on CAF additionally serves as a functional molecule to support the CSC population in the tumor microenvironment. The revealed features of CD44 expression with protein redistribution in the stroma explain the special aggressiveness of PDAC.

The results indicate a significant increase in the expression of the CD44 marker in G3 (Kruskal-Wallis test, p <0.05), which also indicates a link between the marker and the aggressiveness of PDAC. Oncoprotein plays an important role in the adaptive plasticity of cancer cells [8, 9, 11]. Adaptive plasticity of cells is represented by the process of epithelial-mesenchymal transition (EMT), as a result of which cells become more mobile, invasive, and

resistant to apoptosis [6-9, 11]. This plasticity of cells causes the recurrence of the tumor. Numerous studies indicate the survival of CD44 + cells after chemotherapy and radiotherapy and tumor repair at the expense of these cells [1-5].

The association of the CD44 cell surface marker responsible for cell adhesion, with EMT, when cell adhesion is lost, has been studied in some cancers characterized by the EMT phenotype.

It is believed that the functional role of CD44 is pleiotropic, including the induction of EMT, and changes in the cellular cytoskeleton [2, 3, 11]. According to scientific studies, overexpression of CD44 leads to an increase in the phenotypes of mesenchymal cancer cells associated with EMT [6-8]. Moreover, cells that undergo EMT acquire not only mesenchymal but also stem properties, which was first proven in breast cancer [6-8, 11]. That is, EMT induces the expression of stem cell markers. High invasiveness and increased ability to metastasize CD44 + cancer cells are also associated with EMT. CD44 overexpression, according to the literature, may be due to loss of functional p53, leading to loss of control over the cell cycle and disruption of apoptosis [14].

Thus, the results are consistent with the literature on the critical role of CD44 in the initiation, progression, malignancy, recurrence and resistance of PDAC and can be used as a diagnostic marker of CSC PDAC.

According to the literature, the use of combinations of cell surface markers is proposed to improve the quality of CSC identification [1-5]. Therefore, despite the informativeness of CD44, its expression was analyzed in combination with another marker - EpCAM. The obtained expression of EpCAM also indicates a small percentage of CSC in PDAC. The area of EpCAM-positive cells was significantly lower than CD44 + in both study groups ($p < 0.05$). Research has shown a partial association between EpCAM expression and EMT [6-9]. As a molecule of epithelial-cell adhesion, the marker plays an active role in the pathological processes of cell proliferation, polarity, and mobility. In addition, the value of EpCAM in the progression of PDAC is associated with its suppressive effect on immune cells in the tumor [8,9]. Accordingly, as stated in the literature sources, marker expression correlates with the degree of tumor differentiation [1-5]. The presence of individual small immunopositive cancer cells on the periphery of the layers may manifest EMT, which these cells undergo, and indicate their greater aggressiveness. There was no significant difference in the marker depending on the degree of differentiation in the studies, but the minimum was three times higher at G3. However, there is evidence of an increase in the proportion of cell surface markers, including CD44 and EpCAM, with tumor aggressiveness. In studies with G2, CD44

1.3 times exceeds EpCAM, in G3 this ratio increases to 1.4. In addition to data on the stimulating role of EpCAM in PDAC, the literature provides data on the inhibitory effect of the marker on the tumor process [2-4]. This may be due to the lack of a significant increase in EpCAM with the increasing aggressiveness of the process.

Correlation analysis found a weak negative relationship with a moderate degree of differentiation ($r = -0.17$, $p > 0.05$) and a weak positive relationship with a low ($r = + 0.06$, $p > 0.05$) one. Nonlinear change of markers in the literature is associated with aggressiveness and tumor progression, which may be due to phenotypic and functional diversification of CSC PDAC.

Conclusions:

1. CSCs play a critical role in PDAC initiation, progression, and aggression.

2. Pancreatic ductal adenocarcinoma is characterized by a moderate level of expression of cell surface markers (CSC) – CD44 and EpCAM.

3. Decreased level of differentiation is accompanied by a significant increase in CD44 expression and a slight increase in EpCAM expression.

References:

1. Ishiwata, T., Matsuda, Y., Yoshimura, H., Sasaki, N., Ishiwata, S., Ishikawa, N., Takubo, K, Arai, T., Aida, J. (2018). Pancreatic cancer stem cells: features and detection methods. Pathol Oncol Res. 24(4):797-805. https://doi: 10.1007/s12253-018-0420-x.

2. Jo, J.H., Kim, S.A., Lee, J.H. et al. (2021). GLRX3, a novel cancer stem cell-related secretory biomarker of pancreatic ductal adenocarcinoma. BMC Cancer. 18;21(1):1241. https://doi: 10.1186/s12885-021-08898-y.

3. Subramaniam, D., Kaushik, G., Dandawate, P., Anant, Sh. (2018). Targeting Cancer Stem Cells for Chemoprevention of Pancreatic Cancer. Curr Med Chem. 25(22):2585-2594. https://doi: 10.2174/0929867324666170127095832.

4. Ercan, G., Karlitepe, A., Ozpolat, B. (2017). Pancreatic Cancer Stem Cells and Therapeutic Approaches. Anticancer Res. 37(6):2761-2775. https://doi: 10.21873/anticanres.11628.

5. Gaertner, B., Carrano, A. C, Sander, M. (2019). Human stem cell models: lessons for pancreatic development and disease. Genes Dev. 1;33(21-22):1475-1490. https://doi: 10.1101/gad.331397.119.

6. Wu Y, Zhang C, Jiang K, Werner J, Bazhin AV and D'Haese JG (2021) The Role of Stellate Cells in Pancreatic Ductal Adenocarcinoma: Targeting Perspectives. Front. Oncol. 10:621937. https://doi: 10.3389/fonc.2020.621937

7. Zhou, P., Li, B., Liu, F., Zhang, M., Wang, Q., Liu, Y., Yao, Y., Li, D. (2017). The epithelial to mesenchymal transition (EMT) and cancer stem cells: implication for treatment resistance in pancreatic cancer. Mol Cancer. 28;16(1):52. https://doi: 10.1186/s12943-017-0624-9.

8. Ahmad R Safa (2020). Epithelial-mesenchymal transition: a hallmark in pancreatic cancer stem cell migration, metastasis formation, and drug resistance. J Cancer Metastasis Treat. 6:36. https://doi: 10.20517/2394-4722.2020.55**. Epub**

9. Rodriguez-Aznar, E., Wiesmüller, L., Sainz, Jr B., Hermann P. C. (2019). EMT and Stemness—Key Players in Pancreatic Cancer Stem Cells. Cancers (Basel). 11(8): 1136. https://doi: 10.3390/cancers11081136.

10. Razi, E., Radak, M., Mahjoubin-Tehran, M., Talebi, S., Shafiee, A., Hajighadimi, S., Moradizarmehri, S., Sharifi, H., Mousavi, N., Sarvizadeh, M., Nejati, M., Taghizadeh, M., Ghasemi, F. (2020). Cancer stem cells as therapeutic targets of pancreatic cancer. Fundam Clin Pharmacol. 34(2):202-212. https://doi: 10.1111/fcp.12521.

11. Patil, K., Khan, F.B., Akhtar, S., Ahmad, A., Uddin, Sh. (2021). The plasticity of pancreatic cancer stem cells: implications in therapeutic resistance. Cancer Metastasis Rev. 40(3): 691–720. https://doi: 10.1007/s10555-021-09979-x.

12. Gzil, A., Zarębska, I., Bursiewicz, W., Antosik, P., Grzanka, D., Szylberg, Ł. (2019). Markers of pancreatic cancer stem cells and their clinical and therapeutic implications. Molecular Biology Reports. 46:6629–6645 https://doi.org/10.1007/s11033-019-05058-1

13. Valle, S., Martin-Hijano, L., Alcalá, S., Alonso-Nocelo, M., Sainz, Jr. B. (2018). The Ever-Evolving Concept of the Cancer Stem Cell in Pancreatic Cancer. Cancers (Basel). 10(2): 33. https://doi: 10.3390/cancers10020033.

14. Barman, S., Fatima, I., Singh, A. B, Dhawan, P. (2021). Pancreatic Cancer and Therapy: Role and Regulation of Cancer Stem Cells. Int J Mol Sci. 30;22(9):4765. https://doi: 10.3390/ijms22094765.

15. Mu, W., Wang, Z., and Zöller, M. (2019). Ping-Pong—Tumor and Host in Pancreatic Cancer Progression. Front Oncol.; 9: 1359. https://doi: 10.3389/fonc.2019.01359.

16. Askan, G., Sahin, I.H., Chou, J.F. (2021). Pancreatic cancer stem cells may define tumor stroma characteristics and recurrence patterns in pancreatic ductal adenocarcinoma. BMC Cancer.;21(1):385. https://doi: 10.1186/s12885-021-08123-w. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

17. López de Andrés, J., Griñán-Lisón, C., Jiménez, G. Carmen; Jiménez, G.; Marchal C., Juan, A. (2020). Cancer stem cell secretome in the tumor microenvironment: a key point for an effective personalized cancer treatment. Journal of Hematology and Oncology. 13(1):136. https://doi: 10.1186/s13045-020-00966-3. [PMC free article] [PubMed] [CrossRef] [Google Scholar]