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L. Shlapatska [1, *], I. Abramenko [2], M. Zavelevich [1], V. Brichenko [2], A. Chumak [2], L. Buchynska [1]

[1] R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, the National Academy of Sciences of Ukraine, Kyiv, Ukraine
[2] National Research Center for Radiation Medicine, Hematology and Oncology, the National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine

* Correspondence: Email: larisash70@ukr.net

SPIKE PROTEIN OF SARS-COV-2 INCREASES CXCR4 EXPRESSION AND MIGRATION OF BREAST CANCER CELLS IN VITRO

Background. There are some data that viral respiratory infections facilitate metastasis of breast cancer (BC). However, whether the coronavirus SARS-CoV-2 is a trigger for BC progression is not yet clear, as well as the possible mechanisms of its involvement. **Aim.** The work aimed to study the effect of the SARS-Cov-2 spike protein (SP) on the expression profile of components of the CXCL12/CXCR4 key signaling axis of BC, cell adhesion markers CD326, CD54, epithelial cells cytokeratin-18 and β-catenin, and migratory activity in in vitro model system. **Materials and Methods.** The expression profile of the studied markers was detected by flow cytometry after 48 h of incubation of MCF-7 and MDA-MB-231 cells with SP. Marker expression was assessed by the number of positive cells (%) and the level of its expression (the relative index of mean fluorescence intensity of cells, iMFI). Cell migration was analyzed by the scratch assay. **Results.** After SP treatment, a significant increase in CXCR4 expression was detected in the cytoplasm of both MCF-7 and MDA-MB-231 cells. SP caused opposite effects on CXCL12 expression — an increase in the MDA-MB-231 cells and decrease in the MCF-7 cells. In addition, in MCF-7 cells, SP treatment resulted in a decrease in the number of CD54-positive cells, the iMFI of CD326, cytokeratin-18, and a slight increase in the iMFI of β-catenin, while in the MDA-MB-231 cell line, a significant decrease in the iMFI of CD54 was observed. SP accelerated the migration activity of MDA-MB-231 and MCF-7 cells, the effect being more pronounced in MDA-MB-231 cells. **Conclusion.** The immunophenotypic changes in the expression profile of several markers under the influence of SP may indicate the induction of the epithelial-mesenchymal transition in the MCF-7 cells and increased migration activity in both cell lines.

Keywords: SARS-Cov-2, spike protein (SP), CXCR4, CXCL12, CD326 (EpCAM), CD54 (ICAM-1), cytokeratin-18, migration activity, MDA-MB-231, MCF-7 cell lines.

Breast cancer (BC) is the most common cancer in women in the world, including Ukraine. In 2021—2022, BC was detected in 22.2% of women with cancer and accounted for 20.2% of female cancer-related mortality in Ukraine [1]. Given the rising trend, it is expected that in 2050 there will be 3.2 million new BC cases and 1.1 million BC-related deaths per year in the world [2]. Metastasis is one of the

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most important problems in treatment of BC. Its pathogenesis is complex. One of the factors, especially in triple-negative BC, is the CXCL12/CXCR4 interaction [3]. CXCL12 is the stromal cell-derived alpha chemokine that binds to the CXCR4 receptor and plays an important role in embryonic development, hematopoiesis as well as tumor growth and metastasis [4]. Under the influence of CXCL12, intracellular pro-proliferative pathways in BC cells are activated, and cell migration toward CXCL12-rich metastatic sites is enhanced [5]. On the other hand, there is evidence that the viral respiratory infections may trigger an inflammation response and promote BC metastasis [6]. After the pandemic caused by coronavirus SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2), some authors noted an increase in metastatic disease at presentation along with a higher histological grade, more frequent lymphovascular invasion, lower estrogen receptor positivity, and an increasing frequency of invasive ductal histological subtype in women with BC as compared to prepandemic findings [7, 8]. First of all, this may be due to the delays in the diagnosis and treatment of cancer patients because of the lockdown and increased burden on the healthcare system, but the pro-oncogenic role of SARS-CoV-2 cannot be completely excluded. In particular, it is known that SARS-CoV-2 increases CXCR4 expression on neutrophils and monocytes and enhances the production of CXCL12 by bone marrow and endothelial cells [9, 10]. No data on SARS-CoV-2-associated changes in the expression of CXCR4 and CXCL12 on tumor cells, in particular BC cells, have been reported. Thus, further studies are needed to evaluate the potential effects of SARS-Cov-2 and its proteins on BC. Cell lines are traditionally used to study phenotype, genetic, and potential treatment options of BC. The aim of the work was to study the effects of SARS-CoV-2 spike protein (SP) on the expression profile of the BC cell lines of different molecular subtypes (MDA-MB-231 and MCF-7) and their migration ability.

Materials and Methods

Two BC cell lines were used: MCF-7, belonging to the luminal molecular subtype, and MDA-MB-231, representing triple negative subtype [11]. The cell lines were obtained from the Bank of Cell Lines from Human and Animal Tissues of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the NAS of Ukraine. Details of cultivation and treatment of the cell lines with the recombinant SP of SARS-CoV-2 (Sino Biological, N 40592-V08H121) at a concentration of 40 pmol/mL were described earlier [12].

Expression of CXCR4 and CXCL12 was determined using antibodies PA3-305 and 79018, respectively (Thermo Fisher Scientific, USA) on Beckman Coulter DxFLEX (Beckman Coulter, USA) with CytExpert (DxFLEX Software). Additionally, the expression of some markers associated with adhesion, migration, and epithelial-mesenchymal transition (EMT) was studied: CD326 (Vu1D9, IEPOR NASU), CD54 (1H4, IEPOR NASU), β-catenin (12F7, Santa Cruz Biotechnology Inc., USA), and cytokeratin 18 (C-04, IEPOR NASU). FITC-conjugated rabbit anti-mouse polyclonal IgG (Dako Cytomation, USA) and donkey anti-rabbit IgG Alexa Fluor 488 conjugate (Molecular Probes, USA) were applied as secondary antibodies. Expression of CD326 and CD54 was examined on the surface membrane of cell lines, while of CXCR4, CXCL12, β-catenin, and cytokeratin 18 — in the cytoplasm. For cytoplasmic detection of antigens, cells were previously pre-fixed with 2% paraformaldehyde and then permeabilized with 0.2% Triton X-100. Each experiment was performed triply. The percentage of positive cells (M ± m) and the relative index of mean fluorescence intensity of cells (iMFI) were evaluated. iMFI was calculated as the ratio of the MFI of cells reacting with antigen-specific mAbs to the MFI of cells incubated with isotype antibodies.

Cell migration was studied by the scratch assay according to Cory [13]. In particular, MCF-7 (13×10^4 cells/well) or MDA-MB-231 (10×10^4 cells/well) cells were grown in 12-well plates until about 80% confluency in DMEM medium containing 10% FBS, and then incubated for 6 h in DMEM medium with 1% FBS. The scratch/wound was made with a p200 pipette tip, after which the debris was removed, and cells were washed once with 1 mL of the growth medium. The width of the initial scratch (time point 0 h) was recorded immediately after the addition of 1.5 mL of standard cell culture medium to the control samples and an additional 40 μg/mL SP to the experimental samples. An important requirement was to select the initial scratches of the same width

in all wells at the beginning of the experiment. The width of the scratches was subsequently assessed after 20 and 28 h in MDA-MB-231 cells, and after 24, 48, and 72 h in MCF-7 cells. The cell migration was assessed using public-free ImageJ software (version 1.50i, National Institutes of Health, Bethesda, MD, USA; https://imagej.net/ij/) by calculating the area of the slit after the indicated period of time relative to the area of the initial scratch.

The statistical analyses were performed with SPSS software (version 17.0; SPSS, Inc., Chicago, IL). All statistical tests were two-sided and considered to be statistically significant at $p < 0.05$.

Results and Discussion

Expression of selected markers (initial and after SP exposure) in MDA-MB-231 and MCF-7 cells are presented in the Table and Fig. 1. It is already known that the CXCR4 molecules undergo internalization upon interaction with the respective ligand and are primarily expressed in the cytoplasm of BC cells [14, 15]. Similarly, the pattern of CXCL12 expression in BC is nuclear/cytoplasmic [16]. Cytoplasmic expression of CXCR4 and CXCL12 was detected in both studied cell lines, although the intensity of CXCL12 expression and percentage of CXCR4-positive MDA-MB-231 cells was lower than in MCF-7. After SP treatment, the number of CXCR4- and CXCL12-positive MDA-MB-231 cells as well as the levels of CXCR4 and CXCL12 expression increased. The number of $CXCR4^+$ and $CXCL12^+$ MCF-7 cells did not change, but a significant increase in CXCR4 and a decrease in CXCL12 expression levels were detected. In the vast majority of studies, increased CXCR4 expression was associated with a higher risk of metastases and poorer prognosis of BC [17, 18]. At the same time, a decreased CXCL12 expression is a negative prognostic factor of BC (regarding tumor size, lymph node metastasis, TNM stage, Her-2 positivity, and lower overall survival) [19—21]. There are data that the CXCL12/CXCR4 axis mediates EMT in BC cells [5]. Given the revealed dynamic changes in the CXCR4 and CXCL12 expression under SP treatment, we also examined the expression of some markers associated with the motility, invasion, and EMT of malignant cells.

Intact MDA-MB-231 and MCF-7 cells differed significantly in the number of CD54-positive cells and the level of CD54 surface expression (Table). Similar results were obtained by Guo et al. [22], who demonstrated that the CD54 surface density ratio in MDA-MB-231 vs MCF-7 cells was 2,350,000 to 323,000 molecules/cell. In our study, after SP-treatment, a decrease in the number of CD54-positive MCF-7 cells was noted. In contrast to the MCF-7 cell line, the content of CD54-positive MDA-MB-231 cells did not change under SP treatment, but the level of CD54 expression decreased. The evidence suggests that high CD54 (ICAM-1) expression promotes BC cell proliferation, EMT, migration, and resistance to apoptosis, while ICAM-1-neutralizing antibody treatment suppressed BC metastasis in the xenograft mouse model [23, 24]. However, there are some opposite data. In experimental models, ICAM-1-deficient C57BL/6-derived E0771 BC cells developed larger metastatic lesions than their control ICAM-1-positive counterparts, and BC patients with elevated ICAM-1 expression on tumor cells had a longer relapse-free periods and a higher overall survival, especially in the luminal B subtypes [25].

Effect of SARS-CoV-2 SP on the expression of markers in BC cell lines

Cell markers	MDA-MB-231				MCF-7			
	Control		SP-treated		control		SP-treated	
	%	iMFI	%	iMFI	%	iMFI	%	iMFI
CXCL12	79.0 ± 1.5	4.9 ± 1.2	90.0 ± 4.6*	9.3 ± 1.2*	88.0 ± 3.1	12.3 ± 1.0#	92.0 ± 2.6	6.3 ± 0.1*
CD54	93.1 ± 2.3	10.8 ± 1.7	88.0 ± 3.4	5.4 ± 1.8*	24.0 ± 2.0#	2.1 ± 0.1#	12.2 ± 3.8*	1.5 ± 0.2
CD326	11.2 ± 2.2	2.5 ± 0.8	12.0 ± 1.4	2.2 ± 0.7	92.0 ± 3.3#	24.6 ± 3.2#	90.0 ± 4.2	11.3 ± 2.7*
β-catenin	97.7 ± 2.3	12.2 ± 0.2	98.0 ± 1.4	12.4 ± 0.1	94.0 ± 2.1	9.6 ± 0.4#	97.2 ± 1.7	11.2 ± 0.1
Cytokeratin 18	91.7 ± 3.1	10.9 ± 1.8	92.0 ± 2.3	9.4 ± 0.8	92.0 ± 2.1	45.3 ± 3.2#	90.0 ± 1.7	31.6 ± 1.1*

Notes: $^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to MDA-MB-231 cells.

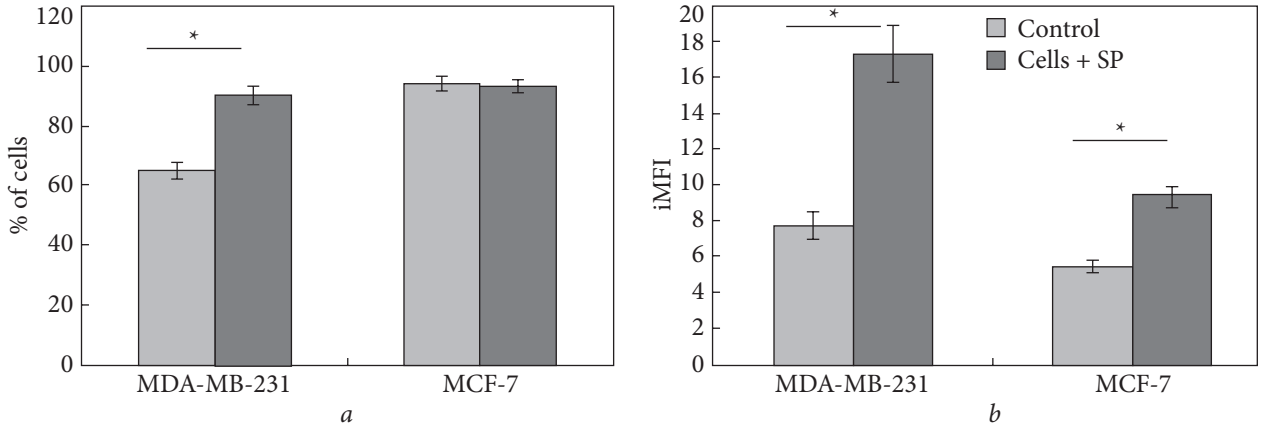
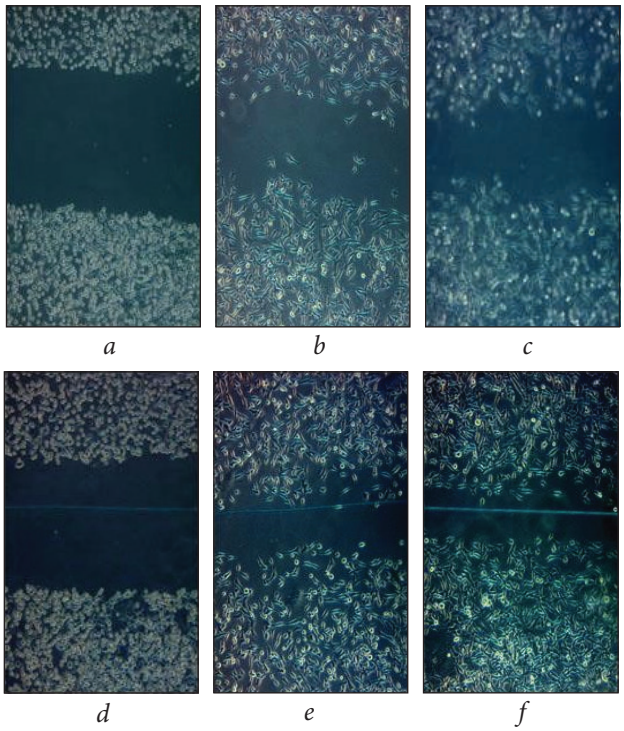


Fig. 1. CXCR4 expression in BC cell lines. Cells were incubated with SP for 48 h. Expression of markers was analyzed by flow cytometry: (*a*) percentage of positive cells, (*b*) level of expression (iMFI). $^{*}p < 0.05$ compared to control



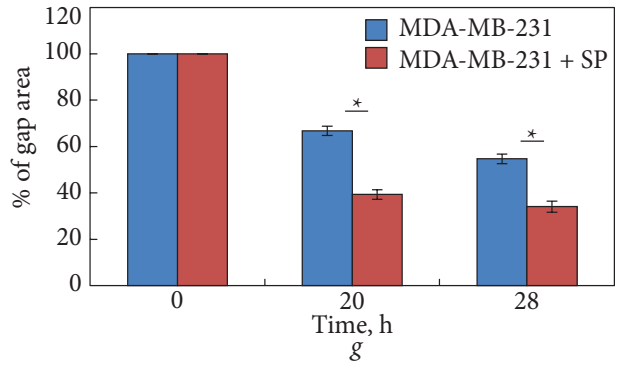
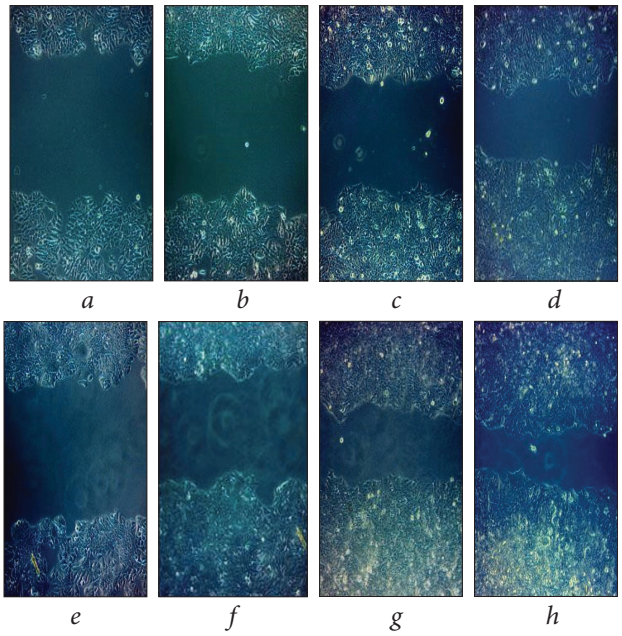


Fig. 2. MDA-MB-231 cell migration upon treatment with SP of SARS-CoV-2. A wound of cell monolayer was made using a p200 pipette tip and registered at the initial timepoint (*a, d*). Cells were further incubated in growth medium with or without 40 μg/mL SP. Cell migration was recorded after 20 h (*b* — control samples, *e* — SP-treated samples) and 28 h (*c* — control samples, *f* — SP-treated samples). The percentage of gap area analyzed by the ImageJ program is presented in a bar diagram showing mean values and standard deviations. The differences between control and SP-treated cells are significant, $^{*}p < 0.05$

CD326 antigen, also known as epithelial cell adhesion molecule (EpCAM), is involved in cellular signaling, proliferation, migration, and differentiation. Numerous data suggest that EpCAM overexpression was associated with poor disease-free and overall survival in BC [26]. In contrast to clinical data, the BC cell lines of more aggressive subtypes reduced expression of EpCAM (namely, MDA-MB-231, triple negative subtype) compared to the cell lines derived from less aggressive subtypes (MCF-7, luminal subtype). The decreased expression of EpCAM on BC cell lines may be associated with EMT. According to the data by Brown et al. [27], in EMT, EpCAM expression is downregulated by extracellular signal-regulated kinases and EMT transcription factors, wherein membrane EpCAM is cleaved, and its intracellular domain moves into the nucleus, stimulating transcription factor genes *OCT4/POU5F1, KLF4*, and *MYC*, which promote growth, cancer stem cell properties. At the same time, some authors believe that the association of EpCAM overexpression with poor prognosis in BC is largely due to CD133 expression [28].

In our study, CD326 expression was detected on the surface of the large fraction of MCF-7 cells, and its expression level was significantly higher in MCF-7 than in MDA-MB-231 cells. These data are in accordance with data of other authors [29, 30].



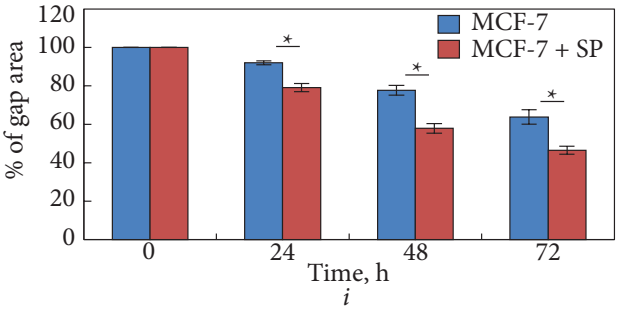


Fig. 3. MCF-7 cell migration upon treatment with SP of SARS-CoV-2. A wound of cell monolayer was made using a p200 pipette tip and registered at the initial time-point (*a, e*). Cells were further incubated in growth medium with or without 40 μg/mL SP. Cell migration was recorded after 24 h (*b* — control samples, *f* — SP-treated samples), 48 h (*c* — control samples, *g* — SP-treated samples), and 72 h (*d* — control samples, *h* — SP-treated samples). The percentage of gap area analyzed by the ImageJ program is presented in a bar diagram showing mean values and standard deviations. * $p < 0.05$

After SP treatment, the number of the CD326-positive MCF-7 cells did not change, but iMFI for CD326 decreased. In contrast, no changes in the CD326 expression profile were noted in SP-treated MDA-MB-231 cells.

Due to the abnormal activation of the Wnt pathway, β-catenin overexpression in BC cells is associated with a low overall survival of patients [31]. In our study, β-catenin was found in the majority of MCF-7 and MDA-MB-231 cells with the same moderate intensity of its expression level. SP did not affect β-catenin expression profile in the cell lines under study.

Cytokeratin 18 was expressed in both studied cell lines, but the expression level was significantly lower in MDA-MB-231 cells. This is in agreement with data showing that cytokeratin 18 down-regulation is associated with histologically poorly differentiated BC, the loss of estrogen and progesterone receptors (that is typical for MDA-MB-231 cells) [32]. The forced down-regulation of cytokeratin 18 in MDA-MB-231 cells enhanced the growth of tumor xenografts in vivo and invasiveness in vitro [33]. In our study, SP treatment reduced the cytokeratin 18 expression level in MCF-7 but not MDA-MB-231 cells.

Summarizing the obtained data, after SP treatment of MDA-MB-231 cells, a significant increase in the number of CXCR4- and CXCL12-positive cells and the iMFI of CXCR4 and CXCL12 expression (with a more pronounced increase in CXCR4) was detected, whereas the iMFI of CD54 decreased. In SP-treated MCF-7 cells, a significant increase in CXCR4 expression, as well as a decrease in the number of CD54-positive cells, and a decrease in the levels of CXCL12, CD326, and cytokeratin 18 expression were observed. Thus, the effect of SP on BC cell lines of different origins was somewhat different.

The decreased CD326 and cytokeratin 18 expression in MCF-7 cells may indicate minor EMT-associated changes evoked by SP. This is in line with data by Lai et al. [34], demonstrating that SP of SARS-CoV-2 induced EMT phenotypic changes in immortal human normal mammary epithelial cell lines (MCF10A and MCF12A) through Snail upregulation. The level of CD326 and cytokeratin 18 expression in MDA-MB-231 cells was initially significantly lower than in MCF-7 cells, and no further decrease occurred under SP treatment. This may be related to the different initial EMT status of these two cell lines. Based on a comprehensive assessment of cell morphology, antigen expression, and motility, MCF-7 cells are considered to possess the epithelial characteristics, while MDA-MB-231 cells seem to have some EMT features [35]. Serwaa et al. [36] also suggested a cell-type-specific manner of the action of SARS-CoV-2. In particular, 22RV1 prostate cancer cells infected by SARS-CoV-2 had an increased proliferative activity, increased expression of Ki-67, BCL-2, vimentin, and the *TNF*-a, *IL*-β, and *IL-8* genes, but down-regulated matrix metallopeptidase 9. In contrast, the proliferative activ-

ity of SARS-CoV-2-infected DLD-1 colorectal cancer cells decreased simultaneously with a decreased expression of all above-mentioned markers; only increased expression of *CXCL8* gene was observed. The significance of down-regulation of CD54 antigen and modulation of CXCL12 under SP-treatment remain unclear.

Common to both studied cell lines was an increase in the CXCR4 expression induced by SP. Since this chemokine receptor is involved in the migration of BC cells in vitro lines and BC metastasis [18, 37, 38], we examined the migratory activity of cells in a scratch assay. The migration rate of MDA-MB-231 cells was higher than that of MCF-7 cells. The cell-free gap in untreated MDA-MB-231 cells amounted to 66.81 ± 2.02% after 20 h and 54.67 ± 2.06% after 28 h, compared to 92.0 ± 0.98% after 24 h, 77.75 ± ± 2.54% after 48 h in MCF-7 cells. Even after 72 h, the cell-free area of MCF-7 cells was 63.75 ± 3.79%. SP treatment accelerated the motility of both MDA-MB-231 and MCF-7 cells. At the end-point of the culture with SP, the wound closure area decreased by 27.5% in MDA-MB-231 cells and by 19.9% in MCF-7 cells compared to the untreated cells (p = = 0.02 and p = 0.016, respectively) (Fig. 2, 3).

Earlier, we did not find any significant short-term effect of SP on the proliferative activity of MDA-MB-231 and MCF-7 cell lines [12]. Furthermore, the phenotype of MDA-MB-231 cells cultured with SP changed toward a decrease in $CD105^+CD90^+$ subpopulation, which may exhibit a stronger migratory capacity compared to $CD105^-/CD90^-$ cells according to the data by Wang et al. [39]. However, despite the reduction of the $CD105^+CD90^+$ subpopulation after SP treatment, the migratory activity of MDA-MB-231 cells increased. We suggest that this may be due to the activation of other factors involved in the migration processes, such as increased CXCR4 expression in both cell lines and CXCL12 in MDA-MB-231 cells. Nevertheless, the presence of the $CD105^+CD90^+$ subpopulation in MDA-MB-231 cells and its absence in MCF-7 cells described in [12] may partially explain the differences in the migratory activity of the cell lines, namely the higher migratory activity of MDA-MB-231 cells.

Thus, in this work, we have shown for the first time an increase in the CXCR4 expression and migratory activity in the MDA-MB-231 and MCF-7 cell lines. This fact raises such provocative questions as the possible boost of metastasis in BC patients after SARS-CoV-2 infection, as well as the safety of using SP-based vaccines in BC patients. Further studies in this direction are needed.

The effects of SARS-CoV-2 on cancer incidence and disease course of cancer patients, including BC, still remain underexplored. Several authors [7, 8] indicated the trend toward recording an increased percentage of more advanced cases of BC with the advent of the COVID-19 pandemic. Different mechanisms of SARS-CoV-2 involvement in cancer pathways have been reviewed to substantiate the oncogenic potential of SARS-CoV-2 [40]. However, recent data demonstrating the positive effect of SARS-CoV-2 spike mRNA vaccination on the sensitization of tumors toward immune checkpoint inhibitors have become evident [41]. The analysis of the molecular mechanisms of the potential carcinogenic and cancer-promoting effects of the SARS-CoV-2 proteins requires comprehensive studies aimed at assessing not only short-term but also the remote effects of SARS-CoV-2 infection and viral proteins on cancer-related cell pathways.

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Л. Шлапацька [1], І. Абраменко [2], М. Завелевич [1],
В. Бріченко [2], А. Чумак [2], Л. Бучинська [1]

[1] Інститут експериментальної патології, онкології і радіобіології ім. Р.Є. Кавецького Національної Академії наук України, Київ, Україна
[2] Національний науковий центр радіаційної медицини, гематології та онкології Національної Академії медичних наук України, Київ, Україна

СПАЙК БІЛОК SARS-COV-2 ПІДВИЩУЄ ЕКСПРЕСІЮ CXCR4 ТА МІГРАЦІЮ КЛІТИН РАКУ МОЛОЧНОЇ ЗАЛОЗИ *IN VITRO*

Стан питання. Вірусні респіраторні інфекції, зокрема спричинені коронавірусом SARS-CoV-2, сприяють метастазуванню раку молочної залози (РМЗ). Проте достеменно не вивчено механізми залучення SARS-CoV-2 у процес прогресування цього захворювання. **Метою** даного дослідження було вивчити вплив спайк білка (SP) SARS-CoV-2 на профіль експресії компонентів однієї з ключових сигнальних ланок при РМЗ CXCL12/CXCR4, маркерів клітинної адгезії CD326, CD54, цитокератину-18, β-катеніну та міграцію в модельній системі in vitro. **Матеріали та методи.** Профіль експресії досліджуваних маркерів вивчали методом протокової цитометрії після культивування клітин ліній MCF-7 та MDA-MB-231 із SP впродовж 48 годин. Експресію маркерів оцінювали за показником кількості позитивних клітин (%) та рівнем їхньої експресії (iMFI). Міграцію клітин вивчали за допомогою методу "подряпини" після 20 і 28 год культивування для клітин лінії MDA-MB-231 та після 24, 48 і 72 год культивування для клітин лінії MCF-7 із SP. **Результати.** Після обробки клітин ліній РМЗ SP виявлено достовірне зростання експресії CXCR4 як у клітинах MCF-7, так і MDA-MB-231, та протилежні відмінності в експресії CXCL12, а саме зростання експресії CXCL12 у клітинах MDA-MB-231 і зменшення в клітинах MCF-7. Окрім цього, у клітинах лінії MCF-7 після взаємодії із SP виявлено зменшення числа CD54-позитивних клітин, рівня експресії CD326, цитокератину-18 та незначне зростання рівня експресії β-катеніну, у той час як у клітинах лінії MDA-MB-231 — достовірне зменшення рівня експресії CD54. Встановлено підвищення міграційної активності клітин ліній MCF-7 та MDA-MB-231 за умов дії SP, яка в клітинах MDA-MB-231 є достовірно вища, аніж у клітинах MCF-7. **Висновок.** Виявлені імунофенотипові зміни профіля експресії ряду маркерів під впливом SP можуть свідчити про індукцію епітеліально-мезенхімального переходу в клітинах MCF-7 та зростання міграційної активності обох клітинних ліній.

Ключові слова: SARS-CoV-2, спайк білок (SP), CXCR4, CXCL12, CD326 (EpCAM), CD54, цитокератин-18, міграційна активність, клітинні лінії MDA-MB-231, MCF-7.