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EXPRESSION OF CHEMOKINE RECEPTOR CXCR4 IN TUMOR CELLS AND CONTENT OF CXCL12$^+$-FIBROBLASTS IN ENDOMETRIOID CARCINOMA OF ENDOMETRIUM

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***Background*: The expression of the CXCL12 chemokine and its receptor CXCR4 in the stromal component of the tumor plays an important role in tumor cell migration, proliferation, inhibition of apoptosis and determination of invasive and metastatic potential of malignant neoplasms of various genesis. The significance of CXCL12 and CXCR4 expression in endometrial tumor cells for cancer progression is not fully understood. *Aim*: To evaluate the content of CXCL12$^+$-fibroblasts and expression of CXCL12 and CXCR4 in endometrial cancer cells, depending on the tumor stage. *Materials and Methods*: Surgical material of 45 patients with endometrioid carcinoma of the endometrium (ECE) of the stages I–II and III was studied using morphological and immunohistochemical methods. *Results*: In ECE of stage I–II CXCR4 expression was lower (43.3 ± 4.2%) while CXCL12 expression was higher (33.6 ± 2.4%) compared with the corresponding indices in ECE of stage III (63.6 ± 3.5%, 24.5 ± 1.9%, respectively, $p < 0.05$). In ECE of stage III, high expression of CXCR4 (> Me) and low CXCL12 (< Me) was observed in 80% of samples; these tumors invaded more than 1/2 of the myometrium. There was a positive correlation between the depth of tumor invasion in the myometrium and the presence of metastases and CXCR4 expression in tumor cells (R = 0.5 and R = 0.4, respectively, $p < 0.05$) and the negative correlation with the expression of CXCL12 (R = –0.6 and R = –0.3, respectively, $p < 0.05$). In tumors that deeply invaded the myometrium, a high number of the CXCL12$^+$-fibroblasts (> Me) (14.9 ± 1.3%) was detected. *Conclusion*: The obtained data reflect the communication of the immunosuppressive factor of the tumor microenvironment, i.e. CXCL12$^+$-fibroblasts and CXCR4 expressing tumor cells. We suggest that the aggressiveness of ECE is determined by the combined effect of these two factors.**
***Key Words*: endometrioid carcinoma, chemokine, chemokine receptor, CXCL12, CXCR4, CXCL12 expressing-fibroblasts.**

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The biological features of a malignant neoplasm are the result of the interaction of tumor cells with the components of tumor microenvironment. An important role in this association is played by chemokines and their receptors, in particular, CXCL12 (also known as SDF-1, stromal derived factor-1) and CXCR4, the expression of which to some extent determines the progression of tumors of different genesis.

The activated CXCL12/CXCR4 axis induces expression of *RAS, PI3K, NF-κB,* and *ERK1/2*, which are components of many signaling pathways (RAS-MAPK, PI3K-AKT-mTOR, NF-κB, MAPK/ERK). They activate matrix metalloproteinases (MMP-2 and MMP-9) and integrins that leads to increase in proliferative potential of tumor cells and inhibition of apoptosis. This all contributes to the increased migratory and invasive activity of cancer cells [1–5]. A known inducer of CXCL12 and CXCR4 expression is the angiogenic factor VEGF. At the same time, CXCL12 can stimulate VEGF expression [1, 2, 6].

The high expression of CXCR4 and CXCL12 in cells of tumor microenvironment, in particular, fibroblasts, correlates with the metastasis of tumors of mammary gland, prostate, lung, colon, ovary, and melanoma [6–8]. At the same time, high expression of CXCR4 and CXCL12 in the epithelial cells of colorectal and breast cancer is associated with a favorable course of the disease [9–11]. In contrast, higher expression of CXCL12 in endometrial cancer (EC) cells is associated with the aggressiveness of the tumor process, whereas the expression of CXCR4 has no prognostic value [12, 13]. Other reports showed that the high CXCL12 and low CXCR4 in EC cells correlated with the favorable course of the disease [14–16], similarly to colorectal and breast tumors.

Therefore, a question of the prognostic value of CXCR4 and CXCL12 expression in EC, which is characterized by clinical polymorphism, remains open. The aim of the present study was to evaluate the number of CXCL12$^+$-fibroblasts and expression of CXCL12 and CXCR4 in EC cells depending on the tumor stage.

MATERIALS AND METHODS

The present study was performed on the surgical material of 45 patients with EC of stages I-II and III (by FIGO), who were treated at the Department of Oncogynecology of the National Cancer Institute of the Ministry of Health of Ukraine in 2014–2019. The patients did not receive preoperative therapy and provided an informed consent on the use of their biological material for the research. According to the conclusion of the commission on bioethics of IEPOR, the study met all the necessary ethical standards,

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Abbreviation used: EC – endometrial cancer; ECE – endometrioid carcinoma of the endometrium; IHC – immunohistochemistry; Me – median; LI – labeling index; PI – proliferation index.

in accordance with the requirements of generally accepted international rules in the framework of the 2008 Declaration of Helsinki.

The morphological diagnosis and the tumor differentiation grade were determined, using hematoxylin and eosin staining of tissue sections, according to the WHO criteria [17].

Immunohistochemical (IHC) detection of biomolecular markers was performed on deparaffinized sections, using primary monoclonal antibodies to CXCR4 (NP PA3-305 "Thermo Fisher Scientific", USA), CXCL12 (clone 79018 "Thermo Fisher Scientific", USA) and PolyVue HRP system/DAB Detection System (Diagnostic BioSystems, USA).

The results of the IHC reaction were evaluated by a semi-quantitative method by counting the number of positively stained cells determined as a percentage — labeling index (LI, %). Marker expression was analyzed in 800–1000 tumor cells. Cell nuclei were stained with Mayer's hematoxylin.

The number of stromal $CXCL12^+$-fibroblasts (%) observed in the proximity to tumor cells was counted in 10 fields of the view at x400 magnification. It should be noted that both the number of tumor cells expressing the proteins and the number of $CXCL12^+$-fibroblasts were analyzed in the same histological specimen.

The proliferative activity of EC cells was assessed by the flow cytometry, using the double staining, with propidium iodide and moAbs to pan-cytokeratin (clone C11, IEPOR); the proliferation index (PI) was calculated as the percentage of cells in the $S + G_2/M$ phases [18]. The study was conducted, using a flow cytofluorimeter EPICS-XL (Beckman Coulter, USA).

The values of LI and PI lower, than the medians (Me) of the corresponding marker were considered as low, and the higher than Me — as high.

Statistical data processing was performed, using a software package STATISTICA 8.0 (StatSoft, Inc.) for non-parametric criteria: Mann — Whitney test, Fisher test (F-test), and a correlation analysis (R — Spearman's rank correlation coefficient). The level of statistical significance was taken as $p < 0.05$.

RESULTS

Morphological verification of the diagnosis revealed that the studied tumors were endometrioid carcinomas of the endometrium (ECE) of moderate (G2) and low (G3) differentiation grade (Table 1). In 64.4% of patients, tumors with deep (> 1/2) invasion in the myometrium were detected, and in 24.4% of cases, stage III ECE and metastasis to regional lymph nodes were detected.

Table 1. General clinical and pathological characteristics of patients with ECE

Index	Number of patients	
	n	%
Total number of patients	45	100
Median age (range), years	60.3 ± 1.4 (32–79)	
Stage		
I–II	34	75.6
III	11	24.4
Metastases		
Present	11	24.4
Absent	34	75.6
Differentiation grade		
G2 (moderate differentiation)	20	44.4
G3 (low differentiation)	25	66.6
Invasion depth		
<1/2 of myometrium	16	35.6
>1/2 of myometrium	29	64.4

According to the results of IHC study, in all studied cases, cytoplasmic and membrane expression of CXCL12 was observed. In most cases, CXCR4 expression was detected in the cytoplasm, but sometimes (8.9%), nuclear localization of this receptor was also observed. Expression of the CXCL12 chemokine and its receptor CXCR4 in tumor cells was detected in the majority of the studied ECE (96.8% and 93.5%, respectively), and $CXCL12^+$-fibroblasts were detected in 93.3% of the neoplasms (Fig. 1).

LI of the CXCL12 expression in tumor cells was 29.9 ± 1.9%, CXCR4 — 49.2 ± 3.5%, and the number of $CXCL12^+$-fibroblasts was 12.2 ± 1.1%. Nevertheless, the individual expression of CXCL12 and CXCR4 in ECE varied in a wide range. The heterogeneity of individual parameters of PI in ECE cells was also demonstrated ranging from 16.5% to 74.0%. It was found that Me of CXCL12 expression was 31.1%, of CXCR4 — 56.4%, Me content of $CXCL12^+$-fibroblasts — 10.7%. The high expression of CXCL12 and CXCR4 (> Me) was observed in 44.4% and 55.6% of cases, respectively. The high content of $CXCL12^+$-fibroblasts (> Me) was detected in 48.8% of tumors.

When assessing the expression of these markers depending on the ECE differentiation grade, we have found that in G3 tumors the number of cells expressing CXCR4 increased compared with G2 tumors (58.5 ± 3.3% *vs* 45.1 ± 5.2%) (Fig. 2, *a*). At the same time, the expression of the CXCL12 was not significantly different in G2 and G3 tumors (30.2 ± 3.0% and 29.7 ± 2.6%, respectively) (Fig. 2, *b*). There was no significant difference in the content of $CXCL12^+$-fibroblasts in tumors of different differentiation grade (10.0 ± 1.8% in G2 tumors *vs* 13.1 ± 1.4% in G3).

No significant differences in PI were found in endometrial tumor cells with low and high CXCL12 expression and with low and high content of $CXCL12^+$-fibroblasts. However, in EC with high expression of CXCR4 there was a significantly higher PI (Table 2).

When assessing the expression of the studied markers in tumors with different depth of myometrial invasion, it was shown (Fig. 3, *a*, *b*) that ECE with invasion > 1/2 of the myometrium was characterized by significantly higher expression of CXCR4 (56.9 ± 4.1%) and a decreased expression of CXCL12 (24.2 ± 2.1%) compared with tumors invading less than 1/2 of the myometrium (35.0 ± 4.7%, $p = 0.04$; 40.1 ± 2.3%, $p = 0.01$, respectively). In addition, in the tumors with deep invasion in the myometrium a higher content of $CXCL12^+$-fibroblasts (14.9 ± 1.3%) was found compared with tumors that invaded less than 1/2 of the myometrium (7.2 ± 1.1%, $p < 0.05$, respectively) (Fig. 3, *c*).

In patients with ECE of stage III significantly higher expression of CXCR4 (63.6 ± 3.5%), lower expres-

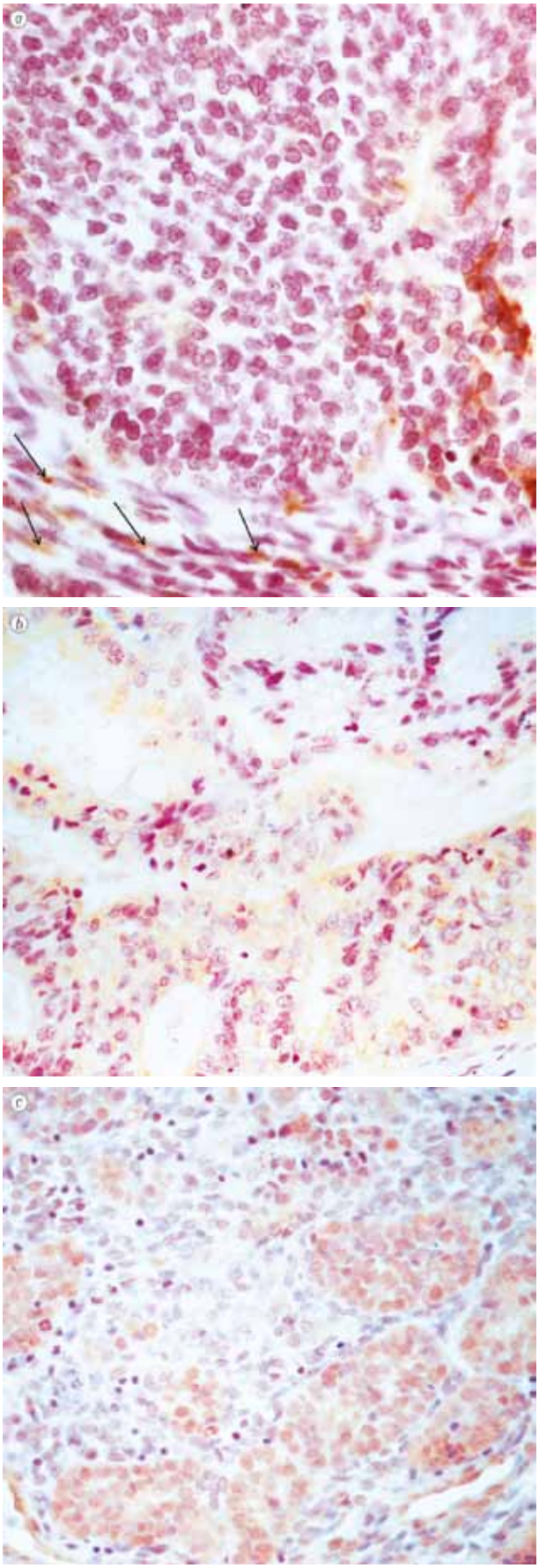

Fig. 1. Expression of chemokine CXCL12 in low differentiated (*a*), and moderately differentiated (*b*) ECE. The arrow indicates stromal CXCL12$^+$-fibroblasts. CXCR4 receptor expression (*c*). IHC method, additional staining with Mayer's hematoxylin. × 400 (*a, c*), × 630 (*b*)

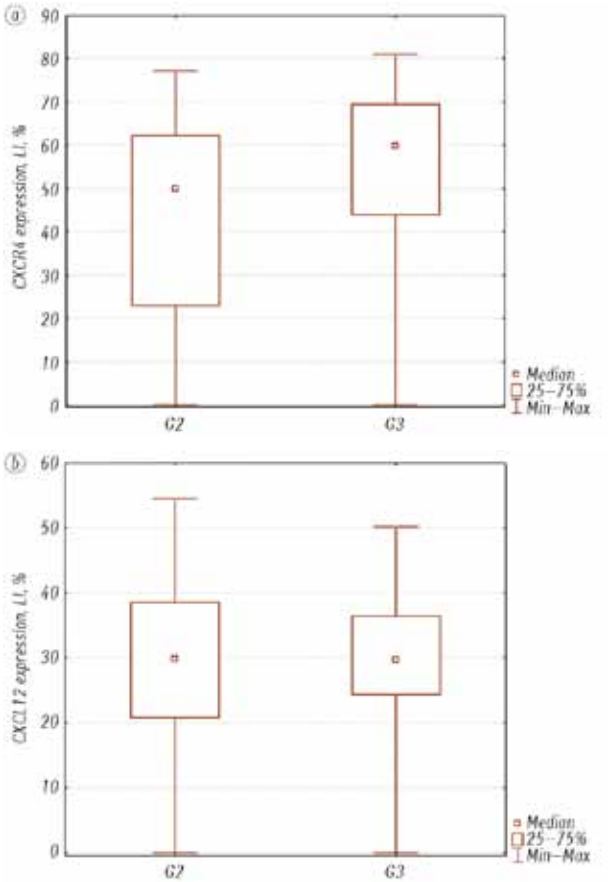

Fig. 2. Expression of CXCR4 (*a*) and CXCL12 (*b*) in ECE cells depending on the tumor differentiation grade

Table 2. Comparison of proliferative potential of ECE with different levels of chemokine receptor expression

Parameter	PI, M ± m,%
CXCL12$^+$-tumor cells:	
< Me	35.9 ± 3.0%
> Me	32.2 ± 2.4%
CXCR4$^+$-tumor cells:	
< Me	28.2 ± 3.9%
> Me	41.1 ± 4.5%*
CXCL12$^+$-fibroblasts	
< Me	33.4 ± 3.4%
> Me	40.0 ± 6.3%

Note: * $p < 0.05$ compared to CXCR4$^+$-tumor cells < Me

sion of CXCL12 (24.5 ± 1.9%) and increased content of CXCL12$^+$-fibroblasts (15.9 ± 2.4%) in tumors were observed compared with these indices in tumors of patients with ECE of stage I–II (43.3 ± 4.2%, $p < 0.05$; 33.6 ± 2.4%, $p < 0.05$ and 10.6 ± 1.1%, $p = 0.05$, respectively) (Fig. 4, *a–c*).

A correlation analysis between such indicators of tumor progression as the depth of tumor invasion into the myometrium and the presence of metastases revealed a positive relationship with the expression of CXCR4 in tumor cells (R = 0.5 and R = 0.4, $p < 0.05$, respectively) and negative correlation with the expression of CXCL12 (R = –0.6 and R = –0.3, $p < 0.05$, respectively).

Therefore, the level of expression of CXCL12 and its receptor CXCR4 correlates with the level of invasion of tumor cells into the surrounding endometrial tissue and the presence of metastases in regional lymph nodes.

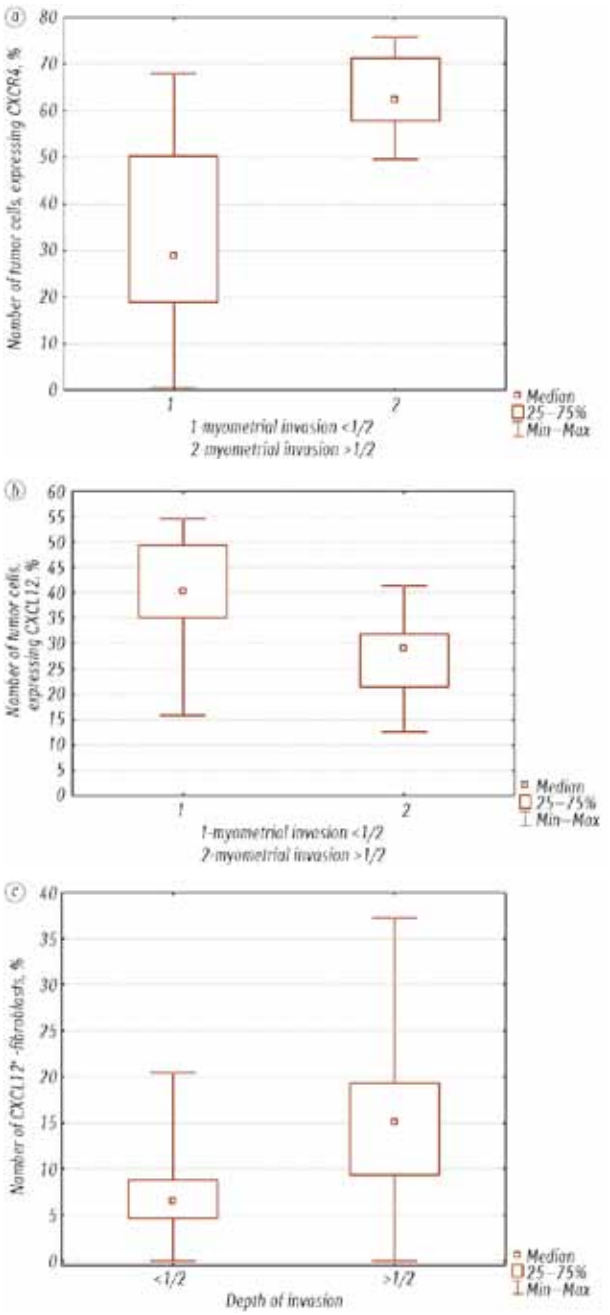

Fig. 3. Expression of CXCR4 (*a*), CXCL12 (*b*) in ECE cells and the content of CXCL12^{+}-fibroblasts (*c*) in the ECE microenvironment depending on the level of tumor invasion into the myometrium

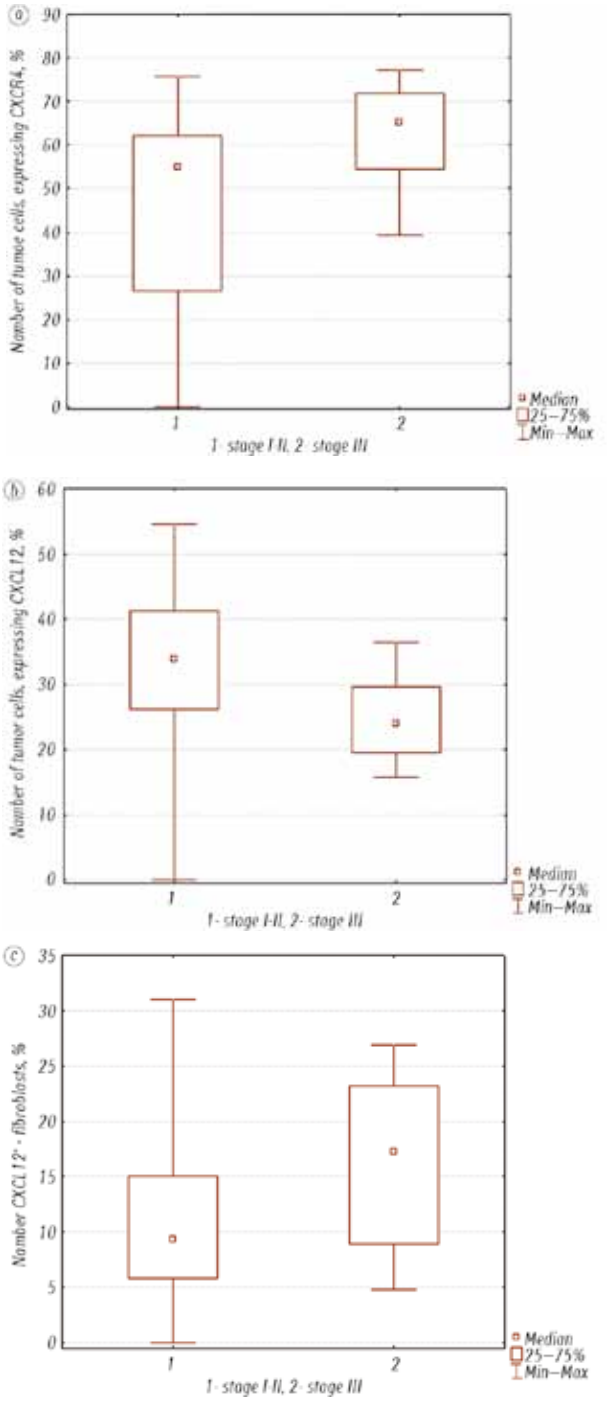

Fig. 4. Expression of CXCR4 (*a*), CXCL12 (*b*) and the content of CXCL12^{+}-fibroblasts (*c*) in ECE depending on the stage of the tumor process

Depending on Me expression of CXCL12 and CXCR4 in the ECE, we have identified the following phenotypic variants of tumors: CXCR4^{high}CXCL12^{low}, CXCR4^{low}CXCL12^{high}, CXCR4^{low}CXCL12^{low}, CXCR4^{high}-CXCL12^{high} (Table 3). As can be seen from the presented data, in patients with ECE of stage III, tumors of CXCR4^{high}CXCL12^{low} phenotype predominated, while neoplasms with the CXCR4^{low}CXCL12^{high} phenotype in this group of patients were absent. In contrast, in patients with stage I–II, the number of tumors with the CXCR4^{low}CXCL12^{high} phenotype increased up to 35.4%. In the group of G3 tumors, CXCR4^{high}CXCL12^{low} and CXCR4^{high}CXCL12^{high} ECs were observed with equal frequency, while tumors with the CXCR4^{low}CXCL12^{low} phenotype were determined only in 16.0% of cases. Instead, ECE with the CXCR4^{low}CXCL12^{high} phenotype predominated among G2 tumors.

ECE with invasion > 1/2 in the myometrium in most (45.0%) cases were characterized by the phenotype CXCR4^{high}CXCL12^{low}. On the contrary, among ECEs that did not deeply invade the myometrium, the CXCR4^{low}CXCL12^{high} phenotype was determined in the vast majority of cases and only 6.2% cases were of the CXCR4^{high}CXCL12^{low} phenotype.

Evaluation of the content of CXCL12^{+}-fibroblasts showed that a significantly higher number of CXCL12^{+}-fibroblasts (15.5 ± 1.7%) was observed in tumors with

Table 3. Analysis of tumor distribution by expression of chemokine receptor CXCR4 and its ligand CXCL12 depending on clinical and pathological characteristics of ECE

Clinical and pathological characteristics of ECE	Tumor distribution by phenotype, n (%)			
	$CXCR4^{high}$ $CXCL12^{low}$,	$CXCR4^{low}$ $CXCL12^{low}$,	$CXCR4^{high}$ $CXCL12^{high}$,	$CXCR4^{low}$ $CXCL12^{high}$,
Stage:				
I–II	8 (23.5)	6 (17.6)	8 (23.5)	12 (35.4)
III	6 (54.5)*	3 (27.3)	2 (18.2)	0*
Differentiation grade:				
G2	6 (30.9)	4 (20.0)	2 (10.0)	8 (40.7)
G3	8 (32.0)	5 (20.0)	8 (32.0)**	4 (16.0)**
Depth of tumor invasion in myometrium:				
<1/2	1 (6.2)	2 (12.5)	4 (25.0)	9 (56.3)
>1/2	13 (45.0)***	7 (24.0)	6 (20.6)	3 (10.4)***

Notes: *$p < 0.05$ compared with tumors of stage I–II, **$p < 0.05$ compared with G2 tumors, ***$p < 0.05$ compared with tumors with invasion <1/2 of the myometrium (F-test).

$CXCR4^{high}CXCL12^{low}$ phenotype compared with the indices in ECs with $CXCR4^{low}CXCL12^{high}$ phenotype (10.0 ± 2.6%, $p < 0.05$), $CXCR4^{low}CXCL12^{low}$ (9.7 ± 2.1%), $CXCR4^{high}CXCL12^{high}$ (12.8 ± 2.1%).

Thus, it is established that the expression of the CXCL12 chemokine and its receptor CXCR4 is associated with certain biological features of ECE of stage I–II and III. ECE of stage III is characterized by decreased expression of CXCL12, increased expression of CXCR4 and increased content of $CXCL12^{+}$-fibroblasts in the microenvironment of endometrial carcinoma, which correlated with a low differentiation grade, deep invasion of the tumor in myometrium and lymph node metastasis.

DISCUSSION

It is known that CXCL12 chemokine and its receptor CXCR4, expressed on the surface of macrophages, lymphocytes, including T-regulatory cells, endothelial and epithelial cells, play a significant role in chemotaxis, invasion, angiogenesis, metastasis and drug resistance of many malignant neoplasms [2, 19–22].

In our study, in patients with stage III disease, high (> Me) CXCR4 expression and low (< Me) expression of CXCL12 were observed in 80% of ECE and these tumors invaded more than 1/2 of the myometrium. In addition, these tumors have a high content of $CXCL12^{+}$-fibroblasts.

It should be noted that TGF-β and S18-2 can induce the CXCR4 expression and this leads to the increased migration capacity of tumor cells [23–26]. In addition, a number of transcription factors can regulate the CXCR4 expression. For example, hepatocyte growth factor (HGF) activates such components of the MAP-kinase cascade as ERK1/2, which, in turn, promotes the expression of NF-κB and HiF-1ά, resulting in the activation of CXCR4 [5, 27].

Undoubtedly, an important factor, involved in regulation of expression, is estrogen: the high content of estrogen induces the expression of CXCR4 and CXCL12 [28]. It is not excluded that the increase in CXCR4 expression found by us in ECE of low differentiation grade occurs due to the high concentration of estradiol in the serum of peripheral blood of the examined patients with ECE [29].

Another mechanism of the regulation of CXCL12 expression depends on epigenetic changes, in particular hypermethylation of its promoter or miRNA31 [30, 31].

According to the literature, tumor-associated $CXCL12^{+}$ -fibroblasts can induce the CXCR4 expression in breast cancer cells [9, 32]. It is important to note that $CXCL12^{+}$-fibroblasts activate Tregs lymphocytes and suppress cytotoxic $CD8^{+}$-lymphocytes in basal-like breast cancer and serous ovarian carcinomas, contributing to immunosuppression, which is one of the factors of the adverse disease course [33–35].

The ratio of the chemokine expression to expression level of its CXCR4 receptor is believed to be essential for the cancer progression [11]. In our study, we found that the phenotype $CXCR4^{low}$ $CXCL12^{high}$ is characteristic for ECE of stage I–II and a lower number of $CXCL12^{+}$-fibroblasts in the stromal component of tumors. In contrast, in the group of patients with ECE of stage III, low expression of CXCL12 and high expression of CXCR4 predominated as well as the increased number of $CXCL12^{+}$-fibroblasts.

It was reported earlier that the high number of $CXCL12^{+}$-fibroblasts correlated with lower overall survival of patients with breast cancer [36]. Moreover, the large number of stromal $CXCL12^{+}$-fibroblasts was associated with the deep invasion of EC into the myometrium metastasizing to lymph nodes and the adverse course of ECE [13].

It was shown earlier that tumor cells expressing the high levels of CXCR4 and low levels of CXCL12 migrated toward tissues with high CXCL12 levels; this correlated with the aggressiveness of the tumor process [9, 10, 30, 37, 38].

Regarding ECE, there are different opinions about the prognostic value of CXCL12 and CXCR4 expression. The higher expression of CXCL12 in EC cells is associated with a higher risk of recurrence and low survival of patients, while no significant values for CXCR4 expression were found [12, 13]. On the contrary, the high CXCR4 expression is associated with advanced clinical stages of EC, deep tumor invasion into the myometrium, lymph node metastases, and a more aggressive tumor phenotype [14–16].

Given the ability of CXCR4-expressing tumor cells for chemotaxis to a gradient of high concentrations of CXCL12-positive cells, it can be assumed that EC cells with high expression of CXCR4 and low expression of CXCL12 can migrate to stroma with a high number of CXCL12 positive fibroblasts. This creates the conditions for the emergence of the locomotor phenotype, determining the invasive and metastatic potential of the tumor [7, 12, 39].

In conclusion, our study showed that the high expression of CXCR4 in tumor cells and the increased number of CXCL12 positive fibroblasts correlate with the deep invasion of ECE cells into the myometrium, the advanced stage of tumor process and the presence

of metastases in lymph nodes, thus justifying their usefullness as prognostic factors for ECE aggressiveness.

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ЕКСПРЕСІЯ ХЕМОКІНОВОГО РЕЦЕПТОРА CXCR4 У ПУХЛИННИХ КЛІТИНАХ І ВМІСТ CXCL12$^+$-ФІБРОБЛАСТІВ В ЕНДОМЕТРІОЇДНІЙ КАРЦИНОМІ ЕНДОМЕТРІЮ

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***Стан питання*:** Важливу роль у процесах міграції пухлинних клітин, їх проліферації, інгібуванні апоптозу та визначенні інвазивного і метастатичного потенціалу злоякісних новоутворень різного генезу відіграє експресія хемокіну CXCL12 та його рецептора CXCR4 у стромальному компоненті пухлини. Питання щодо значення експресії CXCL12 і CXCR4 у пухлинних клітинах ендометрію у прогресії раку ендометрію залишається відкритим. ***Мета*:** Оцінка вмісту CXCL12$^+$-фібробластів та експресії хемокіну CXCL12 і його рецептора CXCR4 у клітинах раку ендометрія залежно від стадії пухлинного процесу. ***Об'єкт та методи*:** Операційний матеріал 45 хворих на ендометріоїдну карциному ендометрію (ЕКЕ) I–II і III стадії пухлинного процесу досліджено з використанням морфологічного та імуногістохімічного методів. ***Результати*:** Показано, що у пухлинних клітинах хворих на рак ендометрію I–II стадії виявляється нижча експресія CXCR4 (43,3 ± 4,2%) і вища — CXCL12 (33,6 ± 2,4%) порівняно зі значеннями цих показників у пухлинах хворих з III стадією пухлинного процесу (63,6 ± 3,5%, 24,5 ± 1,9%, $p < 0,05$ відповідно). Встановлено, що у хворих з III стадією захворювання висока (> Me) експресія CXCR4 і низька (< Me) CXCL12 спостерігалася у 80% ЕКЕ, і ці пухлини інвазували більше 1/2 міометрію. Виявлено позитивний корелятивний зв'язок між глибиною інвазії пухлини в міометрій і наявністю метастазів у хворої та експресією у пухлинних клітинах CXCR4 (R = 0,5 і R = 0,4, $p < 0,05$ відповідно) та негативний — з експресією CXCL12 (R = –0,6 і R= –0,3, $p < 0,05$ відповідно). У пухлинах, що глибоко інвазували міометрій, визначено високий (> Me) вміст таких імуносупресивних компонентів пухлинного мікрооточення, як CXCL12$^+$-фібробласти (14,9 ± 1,3%). ***Висновок*:** Отримані дані відображають комунікацію імуносупресивного фактору пухлинного мікрооточення — CXCL12$^+$-фібробластів та CXCR4$^+$-пухлинних клітин, сукупний вплив яких визначає агресивність пухлинного процесу в ендометрії.

***Ключові слова*:** ендометріоїдна карцинома ендометрію, хемокін CXCL12, рецептор хемокіну CXCR4, CXCL12$^+$-фібробласти.