UDC 616-006.577.112 https://doi.org/10.15407/biotech15.06.055

THE EXPRESSION OF TLR4, IFN-γ, TGF-β AND TNF-α IN THE CELL LINE OF HUMAN SMALL CELL LUNG CARCINOMA NCI-H69 AND IN CISPLATIN-RESISTANT SUBLINE NCI-H69/CPR

I. Stupak[1]
O. Gorbach[2]
O. Sckachkova[2]
N. Senchylo[1]
E. Singayivskiy[1]
M. Starshinova[1]
L. Garmanchuk[1]

[1]Educational and Scientific Centre "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv, Ukraine

[2]National Cancer Institute, Kyiv, Ukraine

E-mail: navi.kaputs@gmail.com
liudmyla_garmanchuk@ukr.net

Received 30.09.2022
Revised 10.11.2022
Accepted 30.12.2022

Aim: to investigate the effect of teichoic acid *Staphylococcus aureus* for expression of pro-inflammatory cytokines and of TLR4 in a human small cell lung carcinoma cell line NCI-H69, and cisplatin resistant subline NCI-H69/CPR .

Methods. Incubation of cells with teichoic acid (1 ng/m) conducted for 2 days. Expression level of TLR4, TGF-β, INF-γ, TNF-α was evaluated by the real time PCR on 7500 Real-Time PCR System, using specific primers and fluorochrome SYBR Green. The reverse transcription reaction was performed with High-Capacity cDNA Reverse Transcription Kit carried out under the conditions: 25 °C — 10 min, 37 °C — 120 min and 85 °C — 5 min.

Results. In cell line culture NCI-H69 addition of teichoic acid increased expression of TLR4 by 1.3 times, and IFN-γ — by 1,1 times. Expression of TGF-β and TNF-α was decreased 2.5 and 4.9 times respectively. In cell line culture NCI-H69/CPR the addition of teichoic acid inhibited the expression of all studied parameters. Expression TLR4 decreased by 4.2 times, IFN-γ — by 1.4 times. Expression TGF-β and TNF-α was depressed 1.6 and 1.2 times. The presented data indicate that teichoic acid of bacterial origin provided the effect of modulating the inflammatory effect in lung cancer cell culture, sensitive and resistant to cisplatin.

Conclusions. Teichoic acid as a ligand of TLR4 modulates the expression of pro-inflammatory and anti-inflammatory cytokines in small cell lung cancer cell culture and suppresses the expression of TLR4 and all investigated cytokines in the cisplatin-resistant cell line NCI-H69.

Key words: teichoic acids; toll-like receptor; pathogen-associated molecular patterns; inflammation; cisplatin; chemotherapy; anti-inflammatory cytokines.

The emergence of cancer immunotherapy has led to a significant breakthrough in treating hematological and solid tumors. Immune checkpoint inhibitors (ICIs) have demonstrated their ability to elicit strong and lasting anticancer responses in patients with cancer [1]. The development of immune checkpoint blockade and chimeric antigen receptor T cell therapies highlights the importance of understanding the fundamentals of tumor immunology, including the role of immunosuppressive tumor microenvironments and tumor neoantigens in shaping cancer development and influencing the effectiveness of treatments [2, 3]. Improving our knowledge of tumor immunology will offer valuable insights into developing more efficient therapies. However, tumors use various

mechanisms, including the production of immunosuppressive cytokines, to evade immune control and limit the success of immunotherapy.

Toll-like receptors (TLRs), which belong to the pattern recognition receptor family, play a critical role in innate immune function and inflammation by detecting pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipopeptides, dsRNA, and bacterial DNA [4, 5].

TLR ligands are often used as adjuvants to increase the immunogenicity of vaccines used in anticancer therapy. For instance, cell wall biopolymers of gram-positive microorganisms Staphylococcus aureus, such as teichoic acids (TAs), are utilized as ligands [6]. Activation of toll-like receptor 4 (TLR4), the first identified human Toll homolog, in immune cells triggers the production of pro-inflammatory cytokines and other mediators against pathogens.

Previous studies have indicated that low serum TLR4 (sTLR4) levels could predict poor survival in early-stage non-small cell lung cancer (NSCLC) patients who underwent surgical resection [3]. Other studies have reported that TLR4 expression is highly increased in lung cancer tissue and positively correlates with lung cancer's malignancy [6].

However, whether TLR4 expression in NSCLC cancer tissue could serve as a prognostic marker needs further investigation. Lung cancer is commonly treated with chemotherapy and surgery. Chemotherapy-resistant cancer cells are a significant obstacle to treating locally advanced or metastatic disease. In modern oncological practice, the search for biologically active compounds that can modulate or enhance the effect of chemotherapy treatment is a critical challenge [7].

In a previous study using the Lewis lung experimental model, TA derived from *Staphylococcus aureus* was found to enhance the proapoptotic and antiproliferative effects of the PO244 bimetallic complex [8]. Similar results were obtained in a study investigating toll-like receptors and proinflammatory cytokines on transplanted Lewis lung carcinoma [9].

The current study aimed to investigate the effect of TA on TLR4 expression and key cytokines in the cell line of human small cell lung carcinoma NCI-H69 and its cisplatin-resistant subline NCI-H69/CPR.

Materials and Methods

Cell line of human small cell lung carcinoma NCI-H69 and cisplatin-resistant subline NCI-H69/CPR (Catalogue number. 91091802) were obtained from "Sigma", Inc. (USA). Cultivation was performed in plastic Petri dishes and 6-well plate (Orange Scientific, Belgium). DMEM medium (Sigma, USA) with 10% fetal calf serum (FBS) (Sigma, USA) and 80 μg/ml gentamicin was used for cultivation. Cultivation was performed in a CO_2 incubator at 95% humidity, 5% CO_2 content and temperature (37 ± 1) °C. Incubation of cells with teichoic acid (1 ng/ml) was performed for 2 days.

TLR-4, TGF-β, INF-γ, TNF-α expression level was evaluated by real-time PCR on 7500 Real-Time PCR Systems ("Applied Biosystems", USA) using specific primers and fluorochrome SYBR Green ("Applied Biosystems", USA). GADPH was used to normalize levels of mRNA for the relative quantification method of analysis. TLR4, TGF-β, INF-γ, TNF-α sequences were constructed by Primer Express® Software v3.0 (Applied Biosystems, USA) (Table). Calculations were performed using the ΔCt relative quantification method. All tests were run in triplicate.

Total RNA was isolated by phenol–chloroform extraction and the "Ribo-zol" kit. RNA concentration in all samples was measured by ThermoScientific NanoDrop-1000 (Thermo Fisher Scientific, USA) and the samples were diluted to 200 ng/μl. cDNA was obtained from total RNA by RT-PCR using "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems, USA). The reverse transcription reaction was run under the following conditions: 25 °C — 10 min, 37 °C — 120 min and 85 °C — 5 min. DNA was diluted - twice with DNA buffer.

Results and Discussions

The expression levels of TLR4, IFN-γ, TGF-β and TNF-α in the human small cell lung carcinoma NCI-H69 and its cisplatin-resistant line are shown in Fig1.

Data presented in Figs. 1 and 2-indicate that the expression level of the TLR4 in the cisplatin resistant cell line is twice higher, than that in NCI-H69. The expression of the IFN-γ is 28 times higher. The expression of the TGF-β and TNF-α has the opposite pattern — their expression is 25 and 16 times higher in NCI-H69 cell line than that in NCI-H69/CPR respectively. The addition of TA was produced tangible effect on the expression of TLR4 and cytokines in the cultures of cells from human lung carcinoma and its cisplatin resistant variant.

In the NCI-H69 cell line culture the addition of TA increased the expression of

Table. **Characteristics of the primers used in the experiment**

Subject of study	Sequence of primers	Annealing temperature of primers (Tm), °C	Concentration of primer, mM
GADPH	f-GCCAAGGTCATCCATGACAACTTTGG r-GCCTGCTTCACCACCTTCTTGATGTC	60	0.25
TLR-4	f- AAATTTCCGCTTCCTGGTCT r- TCAGCCCATATGTTTCTGGA	60	0.25
TGF-β	f –GGACATCAACGGGTTCACTA r –CCGGTTCATGCCATGAATGG	60	0.25
INF-γ	f –CCAACGCAAAGCAATACATGA r-TTTTCGCTTCCCTGTTTTAGCT	60	0.25
TNF-α	f –CCCAGGCAGTCAGATCATCTTC r-AGCTGCCCCTCAGCTTGA	60	0.25

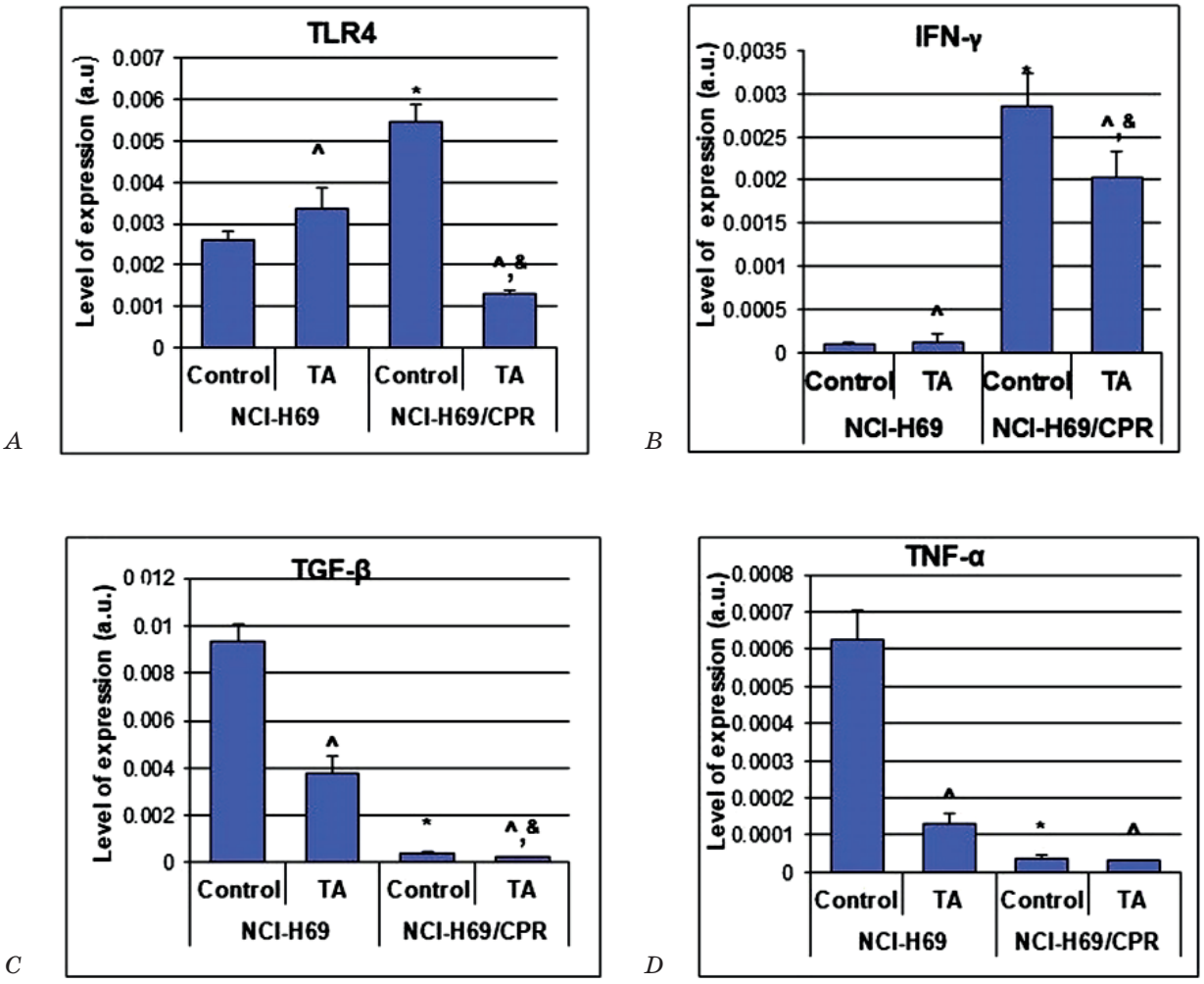


Fig. 1. **Expression level of TLR4 (*A*) and pro- and anti-inflammatory cytokines (*B, C, D*) in human small cell lung carcinoma NCI-H69 and its cisplatin resistant variant cell lines NCI-H69/CPR**
* — $P < 0.05$ *vs* NCI-H69; ^ — $P < 0.05$ *vs* control & — $P < 0.05$ when TA is added to cells NCI-H69/CPR against cells NCI-H69

TLR4 by 1.3 times ($P < 0.05$ *vs* control), and IFN-γ by 1.1 times ($P < 0.05$ *vs* control). The expression of TGF-β and TNF-α was suppressed by 2.5 times and 4.9 times, respectively ($P < 0.05$ vs control) (Fig. 2).

In the NCI-H69/CPR cell line culture the addition of TA suppressed the expression of all studied parameters in the cancer cell culture. The expression of TLR4 decreased by 4.2 times and IFN-γ — by 1.4 times ($P < 0.05$ vs control). The expression of TGF-β and TNF-α was suppressed by 1.6 times and 1.2 times, respectively ($P < 0.05$ vs control).

Lung cancer is the main cause of cancer morbidity and mortality in men, especially smokers, in Ukraine, so the study of this disease is a very relevant topic in oncology. Carcinogenesis involves the host immune system, which uses numerous cytokines and their receptors to control the process, mainly for tumor progression. Internal inflammation in the tissue is an established condition for the initiation of cancer, its progression and the formation of drug resistance. The immune system can play a significant pro- and antitumor role at all stages of tumorigenesis [10]. The complex interplay between cellular and molecular elements of the immune system determines the fate of neoplasia, and on the other hand is a field for potential therapeutic manipulation. Some types of tumor cells have been found to express Toll-like receptors (TLRs) [4, 11], by which immune cells recognize microbial preservative components such as lipopolysaccharide (LPS), teichoic and lipoteichoic acids, and then initiate immune and inflammatory responses by affecting on the expression of such pro-inflammatory cytokines as IFN-gamma, TGF-beta, and TNF-alpha. A change in the expression of markers of the immune system by tumor cells is noted in their resistance to chemotherapy drugs, in particular, such as cisplatin [12]. For the combined therapy of tumors, together with cisplatin , some natural compounds that have an immunomodulatory effect are used [13]. We also observed on the T24/82 bladder cancer cell line that cisplatin in combination with polyphenol compound melanin enhances the antiproliferative effect [14].

In the present study of the effect of TA on the expression of the pro-inflammatory cytokines IFN-γ TGF-β and TNF-α by lung cancer cells sensitive NCI-H69 and resistant to cisplatin NCI-H69/CPR, it was shown that the high level of expression of TNF-α in NCI-H69 cells of the original line was significantly suppressed under the influence of TA, while as in cisplatin-resistant cells NCI-H69/CPR, TNF-α expression was low even without the addition of TA. In contrast to our results, the authors found that upregulation of TNF expression mediated by NF-κB activation plays a key role in resistance to EGFR inhibition in NSCLC, and combined inhibition of EGFR and TNF-α can overcome therapeutic resistance in non-small cell lung cancer [15]. We found a similar effect for both cell lines regarding the expression of TGF-β: a

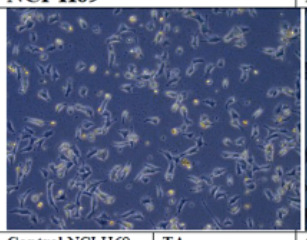
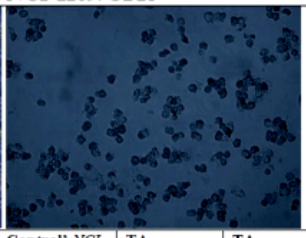
Cells line	**NCI-H69**		**NCI-H69/CPR**		
Changes in cytokine expression					
	Control NCI-H69 cells *Level expression cytokine in the NCI-H69 cells (a.u.)*	**TA** *(Change vs control NCI-H69)*	**Control' *NCI-H69/CPR*** *(Change vs control NCI-H69)*	**TA** *Change vs NCI-H69 cells with TA*	**TA** *Change vs Control' NCI-H69/CPR*
IFN -γ	**0.000102**	$^{\wedge}{}_{1.15}$ ⬆	$^{*}{}_{28}$ ⬆	$^{*}{}_{20}$ ⬆	$^{\#}{}_{1.4}$ ⬇
TGF-β	**0.009355**	$^{\wedge}{}_{2.5}$ ⬇	$^{*}{}_{25.5}$ ⬇	$^{*}{}_{1.6}$ ⬆	$^{\wedge}{}_{16}$ ⬇
TNF-α	**0.000626**	$^{\wedge}{}_{4.8}$ ⬇	$^{*}{}_{16.4}$ ⬇	$^{*}{}_{21}$ ⬇	⬌
TLR 4	**0.002613**	$^{\wedge}{}_{1.3}$ ⬆	$^{*}{}_{2.1}$ ⬆	$^{*}{}_{4.1}$ ⬇	$^{\#}{}_{2.6}$ ⬇

Fig. 2. **Changes in the expression levels of TLR4 and pro-inflammatory cytokines in cells NCI-H69/CPR of the cisplatin-resistant variant of cells against cells NCI-H69 of the original line and under the action of TA**

⬆ — increase; ⬇ — decrease; ⬌ — no change

$^{\wedge}$ — $P < 0.05$ adding TA *vs* control; * — $P < 0.05$ *vs* control NCI-H69; $^{\#}$- — $P < 0.05$ when TA is added to cells NCI-H69/CPR against cells NCI-H69 *a.u.* — *relative units of activity*

high level of expression in cells of the original line NCI-H69 and a significant inhibition when incubated with TA, while in cells resistant line to the action of cisplatin NCI-H69/CPR, a low level of expression of this cytokine was detected both when TA was added and – in control. During studying the expression of TGF-β in the microenvironment of NSCLC cells, the authors found a correlation of this cytokine with the progression, metastasis of lung cancer, and the development of resistance to cytotoxic, targeted and immunomodulatory drugs [16].

It is known that IFN-γ plays a key role in stimulating the antitumor immune response, as this cytokine has a cytostatic, proapoptotic and antiproliferative effect, but its localization in the tumor microenvironment is somewhat controversial and requires more detailed research [17, 18]. We found a decrease in IFN-γ expression upon addition of TA both in the original cell line NCI-H69 and in the cisplatin-resistant cell line NCI-H69/CPR, although the level of IFN-γ expression in the resistant line without the addition of TA was many times higher than in the original line. It is also known that the receptors of the TLR family play a significant role in the progression of lung cancer, in particular, a positive correlation of TLR4 with the differentiation, stage and metastases of lung cancer has been shown [19]. In our study, TA ligand to TLR 4 was shown to have opposite effects on the expression of this receptor in cells of both cisplatin-sensitive and resistant lines of human lung cancer.

Conclusion

The teichoic acid as a ligand for TLR4 modulates the expression of proinflammatory and anti-inflammatory cytokines in cellular culture of small cell lung cancer, and suppresses the expression of TLR4 and all studied cytokines in the cisplatin-resistant strain of NCI-H69/CPR.

Legal entity responsible for the study

This work has been partially supported by the project of the Targeted Training Department of Taras Shevchenko Kyiv National University and the National Academy of Sciences of Ukraine «Study of antitumor properties of biologically active substances of cytokinin nature from the mycelial biomass of medicinal basidiomycetes» (0119U101721).

Disclosure

All authors have declared no conflicts of interest.

REFERENCES

1. *Rizzo A., Mollica V., Santoni M., Massari F.* Cancer Immunotherapy: Harnessing the Immune System to Fight Cancer. *Journal of Clinical Medicine*. 2022. 11 (21), 6356. https://doi.org/10.3390/jcm11216356
2. *Yang Y.* Cancer immunotherapy: harnessing the immune system to battle cancer. *The Journal of Clinical Investigation*. 2015, 125 (9), 3335–3337.
3. *Klener P., Otahal P., Lateckova L., Klener P.* Immunotherapy approaches in cancer treatment. *Current Pharmaceutical Biotechnology*. 2015, 16 (9), 771–781.
4. *Wang K., Wang J., Wei F., Zhao N., Yang F., Ren X.* Expression of TLR4 in Non-Small Cell Lung Cancer Is Associated with PD-L1 and Poor Prognosis in Patients Receiving Pulmonectomy. *Front Immunol*. 2017, V. 8, P. 456. https://doi.org/10.3389/fimmu.2017.00456
5. *Kang X., Li P., Zhang C., Zhao Y., Hu H., Wen G.* The TLR4/ERK/PDL1 axis may contribute to NSCLC initiation. *Internatinal Journal Oncology*. 2020, 57 (2), 456–465. https://doi.org/10.3892/ijo
6. *Kawasaki T., Kawai T.* Toll-Like Receptor Signaling Pathways. *Front Immunol*. 2014, 5, 461. https://doi.org/10.3389/fimmu.2014.00461
7. *Xia G., Kohler T., Peschel A.* The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus. *Journal of Medical Microbiology*. 2010, 300, 148–154 https://doi.org/10.1016/j.ijmm.2009.10.001
8. *Nikulina V., Garmanchuk L., Senchylo N., Nikolaienko T.* Tiechoic acids from *Staphylococcus aureus* enhance cytotoxic/cytostatic influence of bimetallic complex on primary tumor culture. *Journal of Thoracic Oncology*. 2016, 11 (4), 88–89.
9. *Li C., Li H., Jiang K., Li J., Gai X.* TLR4 signaling pathway in mouse Lewis lung cancer cells promotes the expression of TGF-β1 and IL-10 and tumor cells migration. *Bio-medical Materials and Engineering*. 2014, 24 (1), 869-75. https://doi.org/10.3233/BME-130879
10. *Rosenthal R., Cadieux E. L., Salgado R., Al Bakir M., Moore D. A., Hiley C. T., Lund T., Tanić M., Reading J. L., Joshi K., Henry J. Y., Ghorani E., Wilson G. A., Birkbak N. J., Jamal-Hanjani M., Veeriah S., Szallasi Z., Loi S., Hellmann M. D., Feber A., Chain B., Herrero J., Quezada S., Demeulemeester J., Van Loo P., Beck S., McGranahan N., and Swanton C.* Neoantigen directed immune escape in lung cancer evolution. *Nature*.

2019, 567(7749), 479–485. https://doi.org/10.1038/s41586-019-1032-7
11. *Wang K., Wang J., Wei F., Zhao N., Yang F., and Ren X.* Expression of TLR4 in Non-Small Cell Lung Cancer Is Associated with PD-L1 and Poor Prognosis in Patients Receiving Pulmonectomy. *Front Immunol*. 2017, V. 8, P. 456. https://doi.org/10.3389/fimmu.2017.00456
12. *Mansoori B., Mohammadi A., Davudian S., Shirjang S., Baradaran B.* The Different Mechanisms of Cancer Drug Resistance: A Brief Review *Adv Pharm Bull*. 2017, 7(3), 339-348. https://doi.org/10.15171/apb.2017.041
13. *Dasari S., Njiki S., Mbemi B., Yedjou C. G., Tchounwou P. B.* Pharmacological Effects of Cisplatin Combination with Natural Products in Cancer Chemotherapy *Int. J. Mol. Sci.* 2022, 23(3), 1532. https://doi.org/10.3390/ijms23031532
14. *Yakovlev P., Garmanchuk L., FalalyeyevaT., Beregova T., Ostapchenko L., Nehelia A.* A Cytostatic effect of polyphenol compound melanin in combination with Cisplatin on bladder cancer cell line T24/83. *European Urology Supplements*. 2018; 17 (10): e2553–e2554.
15. *Gong K., Guo G., Beckley N., Zhang Y., Yang X., Sharma M., Habib A. A.* Tumor necrosis factor in lung cancer: Complex roles in biology and resistance to treatment. *Neoplasia*. 2021, 23(2), 189–196. https://doi.org/10.1016/j.neo.2020.12.006
16. *Pınar Ö. Eser, Pasi A. Jänne*. TGFβ pathway inhibition in the treatment of non-small cell lung cancer. *Pharmacology & Therapeutics*, 2018, 184, 112–130 https://doi.org/10.1016/j.pharmthera.2017.11.004
17. *Jorgovanovic D., Song M., Wang L., Zhang Y.* Roles of IFN-γ in tumor progression and regression: a review . *Biomarker Research*, 2020, 8 (49). https://doi.org/10.1186/s40364-020-00228
18. *Zaidi M. R., Merlino G.* The two faces of interferon-g in cancer. *Clin Cancer Res.* 2011, 17(19), 1–7.
19. *Gu J., Liu Y., Xie B., Ye P., Huang J., Lu Z.* Roles of toll-like receptors: From inflammation to lung cancer progression. *Biomed Rep*. 2018, 8(2), 126–132. https://doi.org/10.3892/br.2017.1034

ЕКСПРЕСІЯ TLR4, IFN-γ, TGF-β ТА TNF-α В КЛІТИННІЙ ЛІНІЇ ДРІБНОКЛІТИННОГО РАКУ ЛЕГЕНІ ЛЮДИНИ NCI-H69 ТА В ЦИСПЛАТИН-РЕЗИСТЕНТНОЇ СУБЛІНІЇ NCI-H69/CPR

І. Ступак[1], *О. Горбач*[2], *О. Счачкова*[2], *Н. Сенчило*[1], *Є. Сингаївський*[1], *М. Старшинова*[1], *Л. Гарманчук*[1]

[1]Навчально-науковий центр «Інститут біології і медицини»
Київського національного університету імені Тараса Шевченка, Україна
[2]Національний інститут раку, Київ, Україна

E-mails: navi.kaputs@gmail.com, liudmyla_garmanchuk@ukr.net

Мета. Дослідити вплив тейхоєвої кислоти *Staphylococcus aureus* на експресію прозапальних цитокінів і TLR4 у клітинній лінії NCI-H69 дрібноклітинної карциноми легенів людини та резистентній до цисплатину сублінії NCI-H69/CPR.

Методи. Інкубацію клітин з тейхоєвою кислотою (1 нг/мл) проводили протягом 2 діб. Рівень експресії TLR-4, TGF-β, INF-γ, TNF-α оцінювали методом ПЛР у реальному часі на 7500 Real-Time PCR System, використовуючи специфічні праймери та флуорохром SYBR Green. Реакцію зворотної транскрипції проводили за допомогою High-Capacity cDNA Reverse Transcription Kit за таких умов: 25 °C — 10 хвилин, 37 °C — 120 хвилин і 85 °C — 5 хвилин.

Результати. У культурі клітинної лінії NCI-H69 додавання тейхоєвої кислоти підвищувало експресію TLR4 у 1,3 рази, а IFN-γ — у 1,1 рази. Експресія TGF-β і TNF-α була знижена в 2,5 і 4,9 рази відповідно. У культурі клітинної лінії NCI-H69/CPR додавання тейхоєвої кислоти пригнічувало експресію всіх досліджуваних параметрів. Експресія TLR4 знижувалася в 4,2 рази, IFN-γ — в 1,4 рази. Експресія TGF-β і TNF-α була знижена в 1,6 і 1,2 рази. Наведені дані свідчать про те, що тейхоєва кислота бактеріального походження забезпечила ефект модуляції запального ефекту в культурі клітин раку легенів, чутливих і стійких до цисплатину.

Висновки. Тейхоєва кислота як ліганд TLR4 модулює експресію прозапальних і протизапальних цитокінів у культурі клітин дрібноклітинного раку легенів і пригнічує експресію TLR4 і всіх досліджених цитокінів у стійкій до цисплатину клітинній лінії NCI-H69.

Ключові слова: тейхоєві кислоти; толл-подібний рецептор; патоген-асоційовані молекулярні структури; запалення; цисплатин; хіміотерапія; протизапальні цитокіни.