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Evaluation of the functional activity and specificity of the *hSLPI* gene promoter

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ABSTRACT

BACKGROUND: One of the main problems of modern oncotherapy is the lack of selectivity of the antitumor drugs, leading to their systemic toxicity on the body. Targeted expression of therapeutic genes represents a new approach in cancer gene therapy. One of the ways to achieve the selectivity of action of the therapeutic genes towards tumor cells is the use of promoters that are active only in tumor cells, but not in normal cells.

AIM: To evaluate the functional activity and specificity of the promoter of the human secretory leukocyte protease inhibitor *hSLPI*.

MATERIALS AND METHODS: The promoter of the *hSLPI* gene was cloned into the promotorless vector *pTurboGFP-PRL*. The functional activity and specificity of the promoter were assessed by the expression of the mRNA of the *TurboGFP* reporter gene and the intensity of its luminescence in A549, MCF-7, HeLa tumor cells and WI-38 normal cells using real-time polymerase chain reaction with reverse transcription and fluorescence analysis, respectively.

RESULTS: Despite the literature data, the promoter of the *hSLPIa* gene demonstrated high transcriptional activity only against HeLa cervical cancer tumor cells. At the same time, in the cells of lung adenocarcinoma A549 and breast cancer MCF-7, as in normal WI-38 cells, a reduced level of promoter activity was observed.

CONCLUSION: The data obtained confirm the need for a preliminary assessment of the functional activity of tumor-specific promoters in relation to tumor and normal cells.

Keywords: tumor-specific promoter; secretory leukocyte peptidase inhibitor; green fluorescent protein.

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Оценка функциональной активности и специфичности промотора гена *hSLPI*

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АННОТАЦИЯ

Обоснование. Одной из основных проблем современной онкотерапии является отсутствие избирательности действия используемых противоопухолевых препаратов, приводящее к их системной токсичности на организм. Направленная экспрессия терапевтических генов представляет собой новый подход в генотерапии рака. Одним из способов достижения избирательности действия терапевтических генов по отношению к опухолевым клеткам является использование промоторов, которые активны только в опухолевых, но не в нормальных клетках.

Цель — оценить функциональную активность и специфичность промотора ингибитора секреторной лейкоцитарной протеазы человека *hSLPI*.

Материалы и методы. Промотор гена *hSLPI* клонировали в беспромоторный вектор *pTurboGFP-PRL*. Функциональную активность и специфичность промотора оценивали по экспрессии мРНК репортёрного гена *TurboGFP* и интенсивности его свечения в опухолевых клетках A549, MCF-7, HeLa и нормальных — WI-38 методами полимеразной цепной реакции в реальном времени с обратной транскрипцией и флуоресцентного анализа, соответственно.

Результаты. Несмотря на данные литературы, промотор гена *hSLPIa* продемонстрировал высокую транскрипционную активность только в отношении опухолевых клеток рака шейки матки HeLa. При этом в клетках аденокарциномы легкого A549 и рака груди MCF-7, как и в нормальных клетках WI-38, наблюдался пониженный уровень активности промотора.

Заключение. Полученные данные подтверждают необходимость проведения предварительной оценки функциональной активности опухоль-специфичных промоторов в отношении опухолевых и нормальных клеток.

Ключевые слова: опухоль-специфичный промотор; *hSLPI*; *turboGFP*.

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BACKGROUND

Cancer is one of the most common causes of death worldwide in both men and women. Despite advances in chemotherapy, surgery, and radiotherapy, treatment options for advanced cancer are still limited, which include palliative methods only [1]. The standard antitumor therapeutic protocols indicated that surgery and radiotherapy are used to treat local non-metastatic tumors, whereas systemic chemo-, hormonal, and biological therapy are preferably used to treat progressive metastatic tumors. Improved understanding of the molecular biology of tumor cells, the unique characteristics of tumor growth, and the toxicity caused by chemotherapeutic drugs and causing unwanted immense destruction of normal cells of the body have necessitated the search for alternative targeted and effective methods for cancer treatment, with gene therapy being considered the most promising [2].

Gene therapy approaches are characterized by introducing a therapeutic gene into target cells to replace mutant genes associated with carcinogenesis or genes causing the death of tumor cells [3–5]. The first gene therapy constructs, where the therapeutic gene was controlled by constitutive promoters of cytomegalovirus (CMV) or monkey virus, did not have action selectivity and expressed the antitumor agent in all body cells, also exerting a toxic effect on normal cells. The discovery of tumor-specific promoters, hyperactive in certain types of cancer cells and practically inactive in normal cells, enabled to achieve a strict selectivity level in gene therapy drugs [6]. These include regulatory regions of genes encoding proteins hyper-expressed in various types of tumor cells and associated with their malignant transformation, namely, telomerase, apoptosis blockers, epidermal growth factor receptor, homologous recombination proteins, thyroid transcription factor 1, human secretory leukocyte protease inhibitor (hSLPI), carcinoembryonic antigen, and others [2, 7–9]. In addition, promoters are known to be hyperactive at certain stages of carcinogenesis, such as angiogenesis and metastasis [10].

Although the obtained data characterized the specificity and activity of tumor-specific regulatory regions, the cancer cell heterogeneity does not enable to clearly predict the transcriptional activity of a particular promoter region and, consequently, the efficiency of therapy. Therefore, an essential factor in the development of selective gene therapeutic approaches is the selection of an optimal tumor-specific promoter for a specific type of tumor tissue.

AIM

This study aimed to evaluate the functional activity and specificity of the *hSLPI* promoter for subsequent development of a genetic construct that expresses selectively cytotoxic therapeutic genes in tumor cells.

MATERIALS AND METHODS

Bacterial plasmids, strains, and cultivation conditions

The promoter region of the *hSLPI* gene was cloned into the pTurboGFP-PRL vector (Eurogen, Russia) using the *Escherichia coli* NEB5α strain (New England Biolabs, USA). Bacteria were grown in a standard lysogeny broth (LB) medium (peptone 1.5%, NaCl 0.5%, yeast extract 0.5%) at 37°C in a Multitron Standard shaker-incubator (INFORS HT, Switzerland) at 160 rpm. When growing strains carrying plasmids, kanamycin (30 μg/mL) was added to the medium.

To evaluate the transfection efficiency, the pTurboGFP-C plasmid (Eurogen, Russia) was used, where the reporter gene of the green fluorescent protein *TurboGFP* is controlled by the constitutive promoter of the CMV.

Cell lines

For transfection, the tumor cell lines of human lung adenocarcinoma A549, epithelioid carcinoma of the cervix HeLa, and breast adenocarcinoma MCF-7, obtained from the ATCC collection (USA), as well as the cell culture of normal human embryonic lung fibroblasts WI-38, obtained from the "Collection of Vertebrate Cell Cultures" of the Institute of Cytology of the Russian Academy of Sciences were used. The cells were grown at 37 °C in an atmosphere with 5% CO_2 in the standard DMEM medium (PanEco, Russia) supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin/streptomycin, and kanamycin (100 units each).

Polymerase chain reaction (PCR)

The full-length *hSLPI* gene promoter and its fragments were amplified from the genomic DNA of L-68 lung fibroblasts (SibEnzyme, Russia) using high-precision Phusion (Thermo Fisher Scientific, USA) and Encyclo (Eurogen, Russia) DNA polymerases and primers *F-hSLPI-Xho*, *F-hSLPIa-Xho*, *F-hSLPIb-Xho*, and *R-hSLPI-Bam*, with their sequences listed in Table 1. The Tm Calculator service (Thermo Fisher Scientific, USA) was used to calculate the annealing temperature of the primers.

To optimize the PCR conditions, DNA polymerase was selected, and the composition of the reaction mixture (the amount of template, primers, and Mg^{2+} ions) and the amplification mode (duration of each reaction step, primer annealing temperature) were varied. The optimized amplification mode for the full-length *hSLPI* promoter and its fragments is presented in Table 2.

Electrophoresis

Horizontal DNA electrophoresis was performed in 1–1.5% agarose gel in tris-acetate-ethylenediaminetetraacetic

acid (EDTA) buffer (48.4 g/L tris-aminomethane, 11.4 ml/L glacial acetic acid, 100 ml/L 0.5 M EDTA, pH 8.5), at a voltage of 100 V for 30–45 min. To control DNA mobility in the gel and detect DNA in ultraviolet light, 6×DNA-Dye NonTox solution (AppliChem GmgH, Germany) was used. The gel was viewed in ultraviolet light at a wavelength of 365 nm. For an estimate of the DNA fragment lengths, 1 Kb (SibEnzyme, Russia) and 50 bp+ (Eurogen, Russia) DNA markers were used.

Promoter cloning

The resulting *hSLPIa* gene promoter amplification product and the pTurboGFP-PRL vector were hydrolyzed at the XhoI and BamHI restriction sites for 3 h at 37 °C. The PCR product was purified from impurities pre- and post-restriction using the GeneJet PCR Purification kit (Thermo Fisher Scientific, USA). The restricted vector fragments were separated in an agarose gel, and the regions corresponding to the required fragments were excised from the gel and extracted using the LumiPure DNA Gel Extraction Kit (Lumiprobe, Russia). The purified restriction products were ligated at 4 °C for 14–16 h using T4 DNA ligase (Eurogen, Russia).

The heat shock method was used to transform the resulting construct of the chemically competent *E. coli* NEB 5α cells [11]. For transformation, 10 µl of the ligase mixture was added to 100 µl of competent cells. The cells were then subjected to heat shock, incubated on ice for 40 min, and then heated to 42 °C in a water bath for 90 s, after which they were again placed on ice for 2 min. Each sample was supplemented with 600 µl of the LB medium and incubated at 37 °C for 60 min with aeration. The cells were plated on L-agar plates supplemented with kanamycin (30 µg/mL) post-incubation, and kanamycin-resistant clones were selected. The correctness of the construct assembly was confirmed by PCR and restriction analyses and Sanger sequencing.

Cell transfection

For transfection, A549 (10^5 cells/well), HeLa (1.5×10^5 cells/well), MCF-7 (2×10^5 cells/well), and WI-38 (2×10^5 cells/well) cell cultures were inoculated in 24-well culture plates and grown until the cells reached 70–85% confluency. On the next day, the cells were transfected with the created *pTurboGFP-hSLPIa* reporter construct and the *pTurboGFP-C* plasmid and assessed for the transfection efficiency using the Lipofectamine-3000 reagent (Invitrogen, USA) following the manufacturer's protocol. The reagent-to-DNA ratio in the reaction was 3:1, and the amount of DNA in a well was 500 ng. In separate tubes, 1.5 µl of Lipofectamine 3000, 25 µl of Opti-MEM medium (Thermo Fisher Scientific, USA), 500 ng of DNA, and 25 µl of Opti-MEM medium containing 1 µl of the P3000 reagent (Thermo Fisher Scientific, USA) were mixed. The tube contents were combined and incubated for 15 min at room temperature. The resulting mixture was added to the cells and incubated in an atmosphere of 5% CO_2 at 37 °C for 48 h.

Assessment of transfection efficiency

Transfection efficiency was assessed based on the fluorescence intensity of the *TurboGFP* reporter protein in the blue spectrum (480 nm) on an Axio Imager 2.0 fluorescence microscope (Carl Zeiss, Germany). The maximum fluorescence excitation and emission for TurboGFP were 482 nm and 502 nm, respectively.

Isolation of total ribonucleic acid

The total RNA of transfected A549, MCF-7, HeLa, and WI-38 cells was isolated using RiZol reagent (Dia-M, Russia) following the manufacturer's protocol. The concentration of isolated RNA samples was measured using a Qubit 2.0 fluorimeter (Invitrogen, USA). The total RNA obtained was treated with DNase I and heated for 10 min at 75 °C.

Table 1. Primers used in the study

Primer	Sequence 5'→3'
F-hSLPI-Xho	ACTATACTCGAGCTCACTGCAGCCTCAAAC
F-hSLPIa-Xho	ACTATACTCGAGAGATCTCAAATTATCTCACAGCT
F-hSLPIb-Xho	ACTATACTCGAGCTGAGACAACTGAGCTCCAG
R-hSLPI-Bam	AGATAAGGATCCGGTGAAGGCAGGAGTGAC
GFP_F	GAGATCGAGTGCCGCATCAC
GFP_R	GCCTTTGGTGCTCTTCATCTTG
GAPDH_F	CCAGGTGGTCTCCTCTGACTTC
GAPDH_R	CAAAGTGGTCGTTGAGGGCAATG

Table 2. Amplification modes of the full-sized *hSLPI* gene promoter and its fragments

Reaction stages	*hSLPI*	*hSLPIa* / *hSLPb*
Preliminary denaturation	95 °C, 3 min	95 °C, 3 min
Denaturation	95 °C, 10 s	95 °C, 1 min
Annealing	56 °C, 30 s	64 °C, 45 c
Elongation	72 °C, 51 s	72 °C, 1 min
Number of cycles	30	35
Final elongation DNA polymerase	72 °C, 5 min Phusion	72 °C, 10 min Encyclo

Real-time PCR with reverse transcription

The expression level of the *hSLPa* promoter was assessed based on the number of transcripts of the green fluorescent protein gene *TurboGFP*. The RNAscribe RT kit (Biolabmix, Russia) was used to perform reverse transcription reaction. The reaction mixture consisting of 10 ng of total RNA and 2.5 μM of a mixture of random nonanucleotides and oligo(dT)18 (Biolabmix, Russia) was heated at 70 °C for 3 min. Then, a 5×RT buffer and 100 units of RNAscribe RT reverse transcriptase were added to the cooled mixture, incubated at 25 °C for 5 min and at 50 °C for 30 min. The reaction was stopped by heating at 85 °C for 5 min. The resulting cDNA was used for real-time PCR by adding 2.5 μl to a reaction mixture consisting of BioMaster 2×PCR mixture HS-qPCR SYBR Blue (Biolabmix, Russia) and 0.4 μM of forward and reverse oligonucleotides. The oligonucleotide sequences used to amplify the *GAPDH* and *TurboGFP* genes are presented in Table 1. The PCR program included primary denaturation at 95 °C for 5 min and 39 cycles consisting of denaturation (15 s, 95 °C), annealing (15 s, 60 °C), and elongation (20 s, 72 °C). Amplification was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The reaction mode is presented in Table 2.

The obtained PCR data were analyzed using the standard Bio-Rad iQ5 v.2.0 program (Bio-Rad Laboratories, USA). The threshold cycle (Ct) of each sample was determined. The transcript abundance level and the relative mRNA expression in tumor cells were normalized to the expression level of the housekeeping gene *GAPDH*. The *hSLPa* promoter expression level was estimated relative to the constitutive *CMV* promoter expression level in the control. Therefore, the *TurboGFP* expression level was calculated in the control (ΔCtCMVb=CtCMV–CtGADPH) and experimental (ΔCthSlpa=CthSlpa–CtGADPH) samples. Changes in *TurboGFP* expression in the experimental samples relative to the control were calculated using the equation ΔΔCt=ΔCthSlpa–ΔCtCMV. The relative expression level of *GFP* was calculated as 2–ΔΔCt to determine the fold increase or decrease in the expression of the studied gene in the experimental samples relative to the control.

Statistical processing

Statistical processing and visualization of the obtained data were performed in GraphPad Prism 8 (GraphPad Software, USA). Analysis of variance with Tukey's post hoc test was performed for multiple comparisons of several independent samples. The critical significance level for testing statistical hypotheses in this study was p=0.05.

RESULTS

Creation of a genetic construct for the expression of a reporter gene controlled by the *hSLPa* promoter

Regarding the promoter of the human leukocyte secretory protease inhibitor gene *hSLPI*, in addition to the full-length region of 1250 base pairs, its reduced fragments of 877 base pairs (region from –849 to +22) and 684 base pairs (region from –650 to +22), designated as *hSLPIa* and *hSLPIb*, respectively, possess high transcriptional activity [12]. To identify the promoter regions of the *hSLPI* gene, the corresponding primers were used in this study, allowing the amplification of regions of different lengths, differing in 5' regions (Fig. 1*a*). Since the promoter regions, due to the high GC composition, are associated with difficult-to-amplify matrices [13], at stage 1 of this study, the PCR conditions were optimized, namely, the duration of the primary denaturation was increased, the primer and magnesium ion concentrations were varied, the annealing temperature was changed, and a DNA polymerase was selected. The genomic DNA of human lung fibroblasts L-68 was used as a matrix. Consequently, highly specific fragments of *hSLPIa* and *hSLPIb*, approximately 900 base pairs and 700 base pairs in size, respectively, were obtained using Encyclo polymerase with an increase in the primary denaturation of the DNA matrix to 3 min and the magnesium concentration to 2 mM, with annealing temperature of the primers at 64 °C (Fig. 1*b*). The PCR product of the full-length region of the *hSLPI* promoter contained impurity fragments (Fig. 1*b*); therefore, for the next stage of this study, a longer *hSLPa*, with 877 base pairs in size, was selected from the obtained regions of the *hSLPI* promoter.

The *hSLPa* promoter was cloned at the BamHI and XhoI sites into the promoterless *pTurboGFP-PRL* vector containing the reporter gene of the green fluorescent protein *TurboGFP* to assess the functional activity and specificity. The presence of the insert in the resulting plasmid was confirmed by restriction analysis (Fig. 1*c*), and the correctness of the nucleotide sequence of the cloned region was confirmed by Sanger sequencing.

Thus, the genetic reporter construct *pTurboGFP-hSLPa* was created, containing the gene of the green fluorescent protein *TurboGFP* controlled by the *hSLPIa* promoter.

Assessment of the functional activity and specificity of the *hSLPIa* promoter

The specificity of the transcriptional activity of the *hSLPIa* promoter was assessed on tumor cells of human lung adenocarcinoma A549, cervical cancer HeLa, breast cancer MCF-7, and normal human embryonic lung fibroblast line WI-38 using fluorescence microscopy by glow intensity of the green fluorescent protein TurboGFP. Recipient cells were transfected with the created reporter

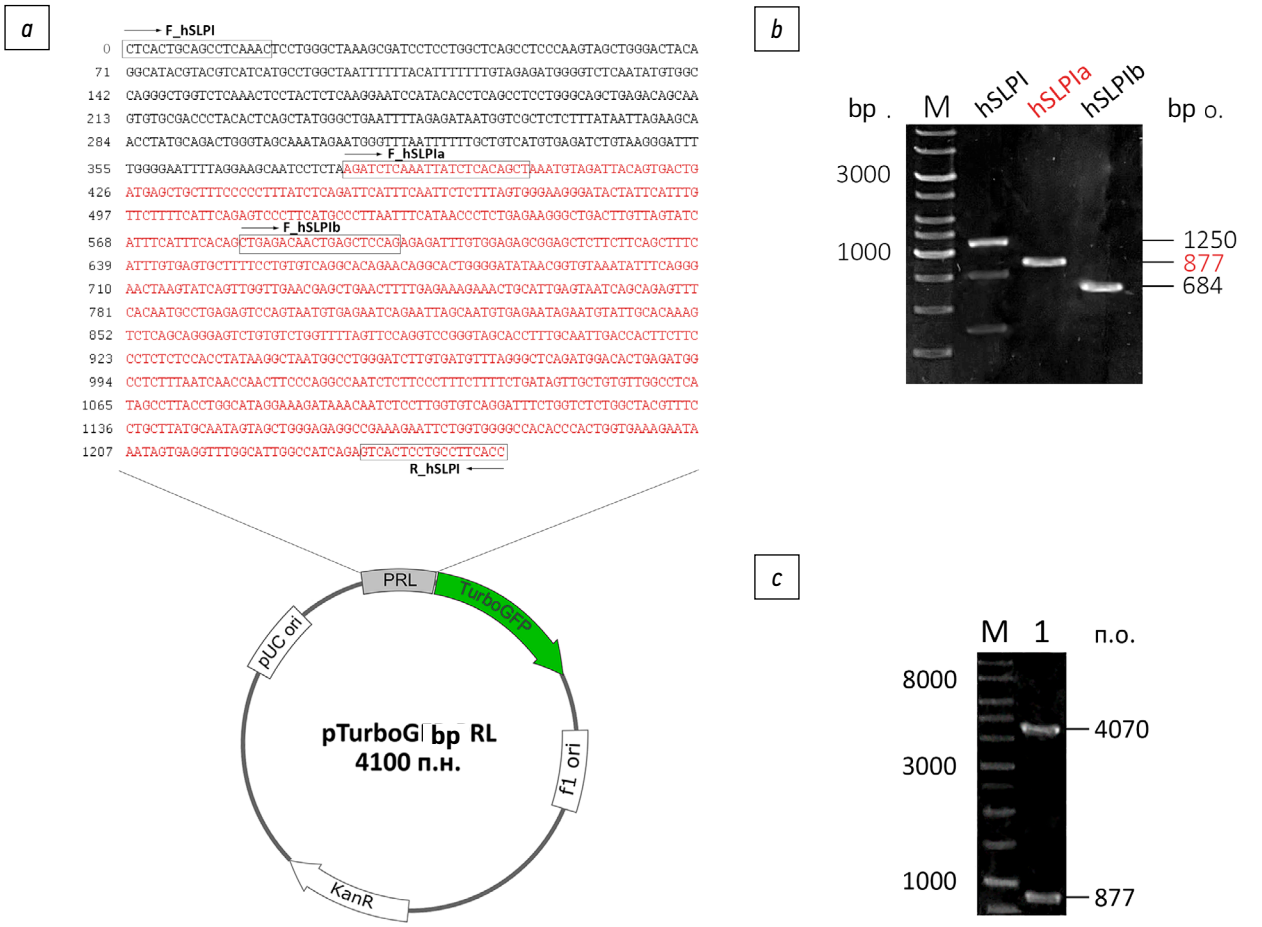

Fig. 1. Cloning of fragments of the *hSLPI* gene promoter into the *pTurboGFP-PRL* vector: *a* — nucleotide sequence of the *hSLPI* promoter fragments and the scheme of the *pTurboGFP-PRL* plasmid; *b* — products of amplification of the *hSLPI* gene promoter fragments; *c* — restriction analysis of the *pTurboGFP-PRL* vector (4070 base pairs) and the *hSLPa* insertion (877 base pairs).

construct pTurboGFP-hSLPa; the pTurboGFP-C vector was used as a positive control, where the *TurboGFP* reporter gene is controlled by the constitutive promoter of the *CMV*, active in all mammalian cells. The lipofection method was used for transfection, and the optimal conditions were selected using the reporter construct pTurboGFP-C. During transfection, a 3:1 lipofectamine-to-DNA ratio was used, with 200 ng/well of DNA in the well.

Fluorescence of the green TurboGFP protein, expressed under the control of the *CMV* promoter, was detected in all cell lines transfected with the *pTurboGFP-C* plasmid (Fig. 2). Simultaneously, when transfecting cells with the created *pTurboGFP-hSLPa* reporter construct, the fluorescence of the *TurboGFP* protein, caused by the *hSLPa* promoter activity, was actively detected only in HeLa cervical cancer cells (Fig. 2). In normal WI-38 cells, the fluorescence signal was detected only in single cells (Fig. 2).

Real-time PCR was used for the quantitative assessment of the functional activity of the *hSLPa* promoter in the created *pTurboGFP-hSLPa* construct based on the mRNA expression level of the *TurboGFP* reporter gene in transfected cells after 48 h of cultivation. The transcriptional activity of the *hSLPa* promoter was calculated relative to the constitutive *CMV* promoter, which is active in all cells. Thus, an increased mRNA expression level of the *TurboGFP* gene was detected in HeLa tumor cells, indicating the highest transcriptional activity of the *hSLPa* promoter in this type of tumor cells (Fig. 3). Simultaneously, in A549 lung cancer and MCF-7 breast cancer tumor cells, as well as in normal WI-38 cells, the mRNA expression level was low (Fig. 3).

Thus, the *hSLPIa* promoter has true functional activity and specificity only in relation to HeLa cervical cancer cells. In other tumor lines evaluated in this study, A549 and MCF-7, the *hSLPa* promoter did not exhibit transcriptional activity. Furthermore, the promoter activity was also not detected in normal WI-38 cells, indicating the possibility of developing a highly selective gene therapy construct based on the *hSLPa* promoter.

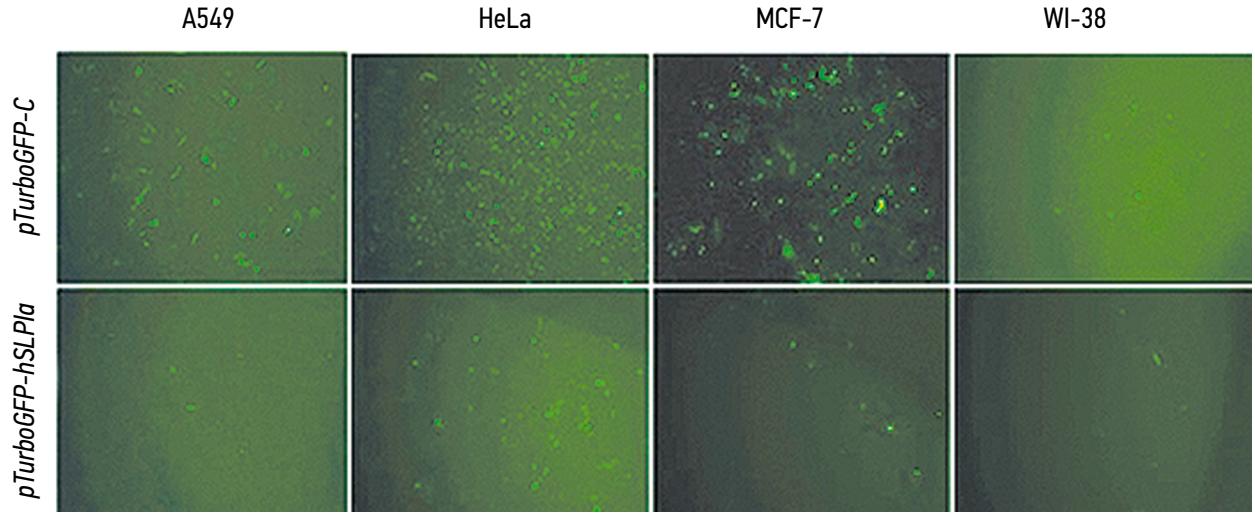

Fig. 2. Fluorescent analysis of the specificity of the *hSLPa* promoter in cells transfected by the *pTurboGFP-hSLPa* after 48 hours of cultivation; magnification ×100.

DISCUSSION

Tumor-specific promoters are one of the promising tools in cancer gene therapy, allowing selective initiation of therapeutic gene expressions exclusively in tumor cells [2]. Currently, several studies have investigated a wide range of promoter regions of genes overexpressed in cancer cells [7–9]. One of the promising ones is the *hSLPI* promoter.

At stage 1 of this study, a reporter genetic construct in which the green fluorescent protein gene is controlled by the *hSLPI* promoter should be created. Promoter regions are complex matrices rich in a large number of GC repeats that are resistant to melting of secondary structures and stop DNA polymerases, which frequently result in incomplete and non-specific amplification [13]. Therefore, a set of primers allowing the amplification of its fragments of different lengths was used to obtain the *hSLPI* promoter. Consequently, the reporter genetic construct *pTurboGFP-hSLPa* was obtained, containing a fragment of the *hSLPI* promoter, 877 base pairs in size (Fig. 1*b*).

The functional activity of the *hSLPI* promoter is known widely vary in range. Hyperexpression of the *hSLPI* gene was revealed in lung cancer cells [14], cervical cancer [15], pancreatic cancer [16], and ovarian cancer [17]. Simultaneously, a low level of *SLPI* gene mRNA expression was detected in the INR-90 normal lung cell line [18]. Tumor cell transfection with the pTurboGFP-hSLPa reporter construct we created showed that the *hSLPIa* promoter can effectively trigger the expression of the fluorescent green protein gene only in HeLa tumor cells (Fig. 2). In the A549 lung cancer and MCF-7 breast cancer cell lines, TurboGFP protein fluorescence was noted only in single cells. Quantitative analysis of the *TurboGFP* gene mRNA

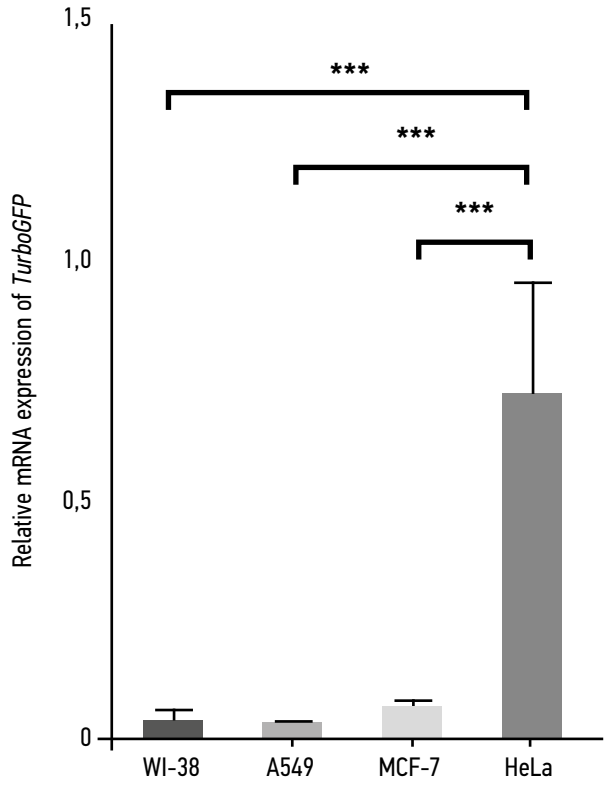

Fig. 3. Analysis of *TurboGFP* mRNA expression from the *hSLPa* promoter in cells transfected by the *pTurboGFP-hSLPa* genetic construct. The expression level of the *TurboGFP* mRNA from the *CMV* promoter in the same cell lines transfected with the *pTurboGFP-C* plasmid was taken as 1; *** p=0.0002.

expression revealed that the activity of the *hSLPIa* promoter in HeLa cells was significantly higher than its activity in both A549 and MCF-7 tumor cells and normal WI-38 cells (Fig. 3). The activity level of the tumor-specific *hSLPIa* promoter in HeLa cells was comparable to that of the non-specific strong *CMV* promoter and was 72% relative to the *CMV* promoter activity in the same cells, which was taken as 100%.

Thus, the functional activity and specificity of the *hSLPI* human leukocyte secretory protease inhibitor promoter was high in HeLa cervical cancer tumor cells, making it a promising tool for creating gene therapeutic constructs for the treatment of cervical cancer.

CONCLUSION

Although several studies have analyzed the activity of tumor-specific promoters, the cancer cell heterogeneity cannot clearly predict the transcriptional activity of a particular promoter region and, consequently, the effectiveness of therapy. Previous studies have reported that the *hSLPI* promoter is active in a wide range of tumor lines. However, its transcriptional activity was detected only in HeLa cervical cancer cells in this study. Therefore, the optimal tumor-specific promoter should be carefully selected for a particular tumor tissue.

ADDITIONAL INFORMATION

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Competing interests. The authors declare that they have no competing interests.

Authors' contribution. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work. Dudkina EV — performed experiments, analyzed and visualized the obtained results, wrote and edited the manuscript; Kosnyrev AS — performed experiments, processed the results obtained and visualized them, wrote and edited the manuscript; Ulyanova VV — analyzed the results obtained, wrote and edited the manuscript, prepared the manuscript for publication; Nadyrova AI — performed experiments, visualized the results obtained, prepared the text of the article for publication; Morozova PS, Kupriyanova EA — performed experiments; Ilinskaya ON — consulted on research, edited the manuscript.

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