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Original paper

The efficiency of silencing expression of the gene coding STAT3 transcriptional factor and susceptibility of bladder cancer cells to apoptosis

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Aim of the study: Abnormalities in signaling as well as altered gene expression have been identified in numerous diseases, including cancer. The biological functions of signal transducer and activator of transcription 3 (STAT3) are very broad. It is thought that STAT3 can also contribute to oncogenesis. RNA interference (RNAi) is one of the most efficient tools for silencing gene expression within cells. The main goal of the study was to verify the effectiveness of STAT3 gene silencing and its influence on cell proliferation and activation of apoptosis in bladder cancer cells.

Material and methods: The study was conducted on cellular material, which was the stable human bladder cancer cell line T24. The synthesis of shRNA (short hairpin RNA) interfering with the STAT3 gene was based on pSUPER. neo expression vector. The gene expression at the mRNA level was determined by the real-time PCR method. The influence of STAT3 gene silencing on apoptosis induced in cells with modulated STAT3 expression was evaluated using parallel quantification of mono- and oligonucleosomal DNA degradation of genomic DNA.

Results: In transfected T24 cells, the STAT3 mRNA expression decreased to the level of 68.3% compared to the scrambled (SCR) control. Silencing the STAT3 gene induced changes in the phenotype of T24 cells. Statistically significant differences in cell proliferation (p = 0.0318) and apoptosis induction (p = 0.0376) were observed.

Conclusions: Application of the designed shRNA for the STAT3 gene contributed to a decrease of expression of the examined gene. It also decreased the proliferation and increased the susceptibility to apoptosis in T24 bladder cancer cells.

Key words: RNA interference, shRNA, signal transducers and activator of transcription, apoptosis.

Introduction

Despite the systematic development of diagnostics and treatment of many neoplastic diseases, there is still an unresolved problem with controlling and blocking the excessive proliferation of cancer cells. This process is connected with increase or constitutive expression of individual genes in the cancer cells. The most often overexpressed genes are those which are involved in survival, intensive proliferation and cells' defensive system, for example genes which control the process of apoptosis.

One of the most essential factors in the process of carcinogenesis is STAT proteins (signal transducer and activator of transcription). In normal human cells, STAT proteins are inactive, cytoplasmic transcriptional factors, which can be activated by extracellular signal molecules, for example growth factors, cytokines, hormones and peptides [1–3].

STAT3 signaling pathways play an important role in physiological processes, such as organogenesis, embryogenesis, innate and acquired immune response, regulation of differentiation of the cells, growth and apoptosis [4–10]. Among STAT proteins, *STAT3* has a pleiotropic nature and can be activated by many cytokines, including interleukin 6 (IL-6), IL-10 and interferon I (IFN I). Depending on cell type, *STAT3* can lead to apoptosis or can promote cell survival. The key role of *STAT3* in the process of carcinogenesis is connected with the fact that overexpression of STAT proteins can indirectly lead to the malignant transformation and expansion of cancer cells, through intensive proliferation and apoptosis inhibition [11]. Limiting or completely silencing the expression of the genes coding transcriptional factors seems to be a promising strategy in anticancer therapy.

The silencing of gene expression can be done on both transcriptional and post transcriptional levels. RNA interference (RNAi) is an innovative technique which creates an opportunity for using nucleic acids as anticancer drugs, which can post transcriptionally suppress the expression of selected target genes [12]. Using RNAi, activation of the molecular degradation of selected transcripts is possible. The process is based on intercellular amplification of short, antisense, ribonucleic sequences [13].

In the present work, the RNAi phenomenon has been used for post transcriptional silencing of expression of the gene coding the *STAT3* protein. We have also examined the susceptibility of bladder cancer cells, transfected with gene-specific siRNAs (small interfering RNAs), to apoptosis in the presence of topoisomerase-etoposide inhibitor.

Material and methods

Designing shRNA and cloning it into vector

The target interfering sequence for shRNA, silencing the selected gene (*STAT3*), was designed using programs from the EMBOSS software package [14]. From a group of designed sequences we chose one which fulfilled the following requirements: the chosen sequence was localized about 50 to 100 nucleotides above the initiation of the translation region; we avoided regions located near the start codon (AUG) and noncoding sequences (5' or 3' UTR, *untranslated region*), which are usually rich in sequences binding regulatory proteins. As a result, the RISC complex has difficult access to target mRNA and efficiency of the whole process could be low; the analyzed sequence should contain 30–50% of G/C pairs; sequences rich in guanine were avoided, as they create quaternary structures. Furthermore, it was checked whether chosen sequences were complementary as regards other genes. In this case, we confirmed that the designed sequence would silence only our chosen *STAT3* gene [15].

The designed sequence for *STAT3* shRNA was cloned into pSUPER.neo vector (OligoEngine) and verified according to the manufacturer's procedure [16]. Preparing and multiplying plasmid DNA containing the shRNA sequence took place in a culture of competitive *Escherichia coli* bacteria. After verification of correctness of the cloning using enzymatic hydrolysis and dideoxy sequencing, the designed shRNA silencing the *STAT3* gene was used for transfection of human eukaryotic cell line T24 (transitional urinary bladder cancer cell line ATCC: HTB4).

Cell cultures and silencing expression of *STAT3* gene

The human cancer cell line T24 culture was cultivated in standard conditions (temperature 37°C, humidity 95%, atmosphere containing 95% air and 5% CO_2). The culture was cultivated in the Hera-Cell incubator (Heraeus). Cells were grown in RPMI-1640 medium containing L-glutamine (PAA Laboratories GmbH), with the addition of 10% FBS (fetal bovine serum) (PAA Laboratories GmbH). The doubling time of the cells in the culture was approximately 22 hours. The condition of the cell culture was measured using microscopic analysis (Axiovert inverted microscope, Zeiss). The cells' lifespan was measured in the Neubauer hemocytometric chamber using 0.1% trypan blue in physiological saline (Sigma).

Transfection of the cells was carried out with lipofection technique, using Lipofectamine® factor. The experiment was conducted according to the producer's guidelines (Invitrogen).

Evaluation of RNA interference at the transcript level

The *STAT3* mRNA expression level in the native and transfected cells was evaluated using the real-time RT-PCR method based on commercially available probes and primers for amplification reactions (Applied Biosystems). For calculations, the Ct technique was used. As the reference gene, β-actin was applied. The control culture consisted of non-transfected cells from the T-24 cell line and the cells transfected with interfering, nonsense sequence SCR (*scrambled*), which was not complementary to any of the human genes.

Evaluation of proliferation and apoptosis activation in cancer cells

In order to check how silencing the expression of the *STAT3* gene changes the susceptibility of transfected cells to apoptosis, a qualitative method (DAPI nucleic acid staining) and quantitative evaluation of the degradation level of genomic DNA were carried out. For this purpose, an immunoenzymatic technique of detection of internucleosomal products of apoptotic DNA decay, Cell Death Detection ELISA Plus Assay (Roche Applied Science), was used. As the apoptosis inducer, the topoisomerase-etoposide inhibitor in the concentration of 20 μM was applied [17]. To examine changes in the cell proliferation rate the MTT assay was performed.

Results

The siRNA sequence silencing *STAT3* expression was designed using the SIRNA program from the EMBOSS software package [14]. As an initial file we used mRNA for the *STAT3* gene from the GenBank database [15] with reference number NM_003150 (4953 nucleotides in length).

The program suggested 274 silencing sequences with the score in the range 10–1. After analyzing all suggested sequences above 9 scores and above 50% of GC (guanine-cytosine) pairs, the sequence 5'aagattgacctagagacccactc 3' was chosen.

The chosen sequence has shown complementarity to all three known transcriptional variants of the *STAT3* gene of the human genome:

- to the mRNA sequence of the third transcriptional variant of the human *STAT3* gene (reference number NM_213662.1):

```
Query  1     AAGATTGACCTAGAGACCCACTC  23
             |||||||||||||||||||||||
Sbjct  1483  AAGATTGACCTAGAGACCCACTC  1505
```

- to the mRNA sequence of the second transcriptional variant of the human *STAT3* gene (reference number NM_003150.3):

```
Query  1     AAGATTGACCTAGAGACCCACTC  23
             |||||||||||||||||||||||
Sbjct  1569  AAGATTGACCTAGAGACCCACTC  1591
```

- to the mRNA sequence of the first transcriptional variant of the human *STAT3* gene (reference number NM_139276.2):

```
Query  1     AAGATTGACCTAGAGACCCACTC  23
             |||||||||||||||||||||||
Sbjct  1591  AAGATTGACCTAGAGACCCACTC  1613
```

In the description above, the phrase *Query* means the designed sequence of interfering RNA and the phrase *Sbjct* means the fragment complementary to all transcriptional variants (1–3) of mRNA for the *STAT3* gene. Based on the chosen sequence, proper oligonucleotide constructions were

GATCCCCGATTGACCTAGAGACCCACTTCAAGAGAGTGGGTCTCTAGGTCAATCTTTTTA
GGGCTAACTGGATCTCTGGGTGAAGTTCTCTCACCCAGAGATCCAGTTAGAAAAATTCGA

Fig. 1. Designed and created sequence used for creating the pSUPER vector, which actively expresses shRNA molecules complementary to STAT3 mRNA. The sequences recognized by appropriate restriction enzymes and used for cloning designed shRNA to the pSUPER vector are marked in red. Target siRNA sequence is marked in blue, nonsense sequence is marked in green, and the loop sequence of final shRNA is marked in violet

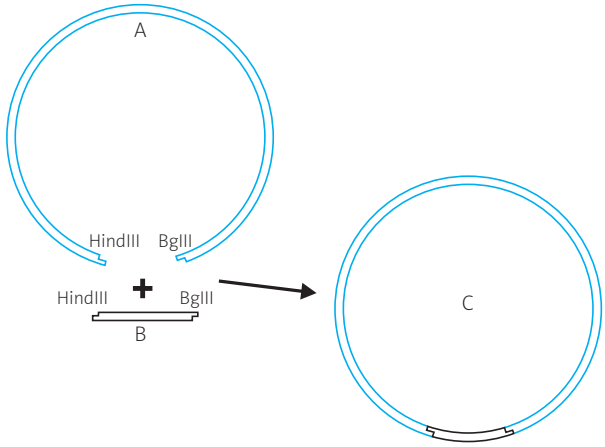

Fig. 2. The process of cloning the insert to the linearized plasmid pSUPER.neo. A – linearized plasmid with sticky ends after digestion with HindIII and BglII enzymes, B – insert obtained by hybridization of oligonucleotide forward and reverse shRNA having sticky ends complementary to the ends of the plasmid after linearization; C – ligation product used for shRNA expression

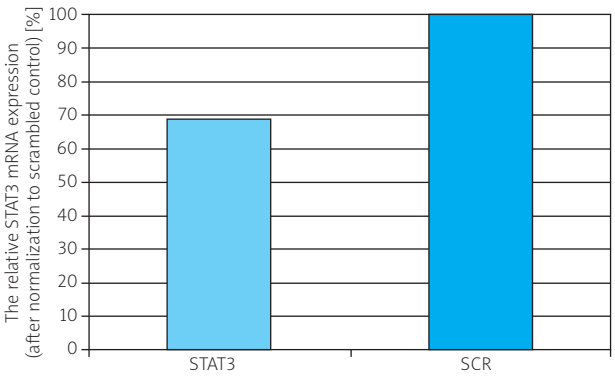

Fig. 3. Changes in the expression level of mRNA for STAT3 gene in the T24 cell culture 24 hours after inserting the sequence silencing STAT3 gene with reference to control culture (SCR)

designed. These constructions included both sense strain and antisense strain of the target gene. Additionally, for cloning purposes, the nucleotide systems were added. As a result, the final construction included restriction sites and the element determining the creation of short hairpin RNA conformation (shRNA). As the cloning was conducting in the plasmid DNA vector, the design of a double strained structure including *forward* and *reverse* oligonucleotides was taken into consideration. The *forward* and *reverse* oligonucleotides corresponded to sense and antisense fragment sequences of the targeted gene.

All designed sequences including the silencing region of mRNA for *STAT3* are described below:

- oligonucleotide *STAT3*shF (*forward*):
  5'GATCCCCGATTGACCTAGAGACCCACTTCAAGAGAGTGGG TCTCTAGGTCAATCTTTTTA 3'

content of GC pairs: 46.7%; molecular weight: 18366.00 g/mol; TM: 72.8°C;

- oligonucleotide *STAT3*shR (*reverse*):
  5'AGCTTAAAAAGATTGACCTAGAGACCCACTCTCTTGAAG TGGGTCTCTAGGTCAATCGGG 3'

content of GC pairs: 46.7%; molecular weight: 18 464.00 g/mol; T_M: 72.8°C.

As a result of hybridization of 60-nucleotide forward and reverse sequences, a double strained oligonucleotide was created (Fig. 1). This oligonucleotide had so-called "sticky 5' ends" with a sequence complementary to the cloning site in the pSUPER.neo vector (Fig. 2) and to the *STAT3* transcript. The *STAT3* transcript binds to the region from 1569 to 1591 nucleotides.

In order to evaluate the gene expression at the mRNA level, the method of indirect characterization of gene expression was used. This method compares the number of mRNA copies of the examined gene to the number of mRNA copies of the β-actin gene, which is used as an endogenic control. 24 hours after cell transfection with the shRNA construction for *STAT3* and with the control construction (shRNA-SCR), the level of mRNA expression for the *STAT3* gene was assessed using real-time RT-PCR. Using the ΔΔCt method of calculations we established that the mRNA expression level for the *STAT3* gene in the T24 cell line (with silenced *STAT3*) was on average at the level of 68.3% of the mRNA expression level of *STAT3* in the T24 cell line transfected with the shRNA-SCR construction (Fig. 3).

With reference to phenotypic changes which are the result of silencing of *STAT3* transcript activity, the evaluation of proliferation and apoptosis induction changes in the T24 cells was carried out. The cells which were transfected with the pSUPER vector including the sequence silencing expression of mRNA for the *STAT3* gene were compared to the cells which were not transfected. Figure 4 shows the T24 cells stimulated to apoptosis with etoposide and stained with DAPI fluorescent dye. In the group of non-transfected cells (A) apoptotic bodies were not observed. In the group of cells with silenced *STAT3* gene stimulated to apoptosis with etoposide (D) the highest number of apoptotic bodies was observed in comparison to other experimental groups of cells (B,C). The increase of apoptosis activation was also assessed quantitatively based on the increase of the amount of internucleosomal cleavage of genomic DNA of transfected cells, which were stimulated with topoisomerase-etoposide inhibitor. In analogy to the result obtained through fluorescent

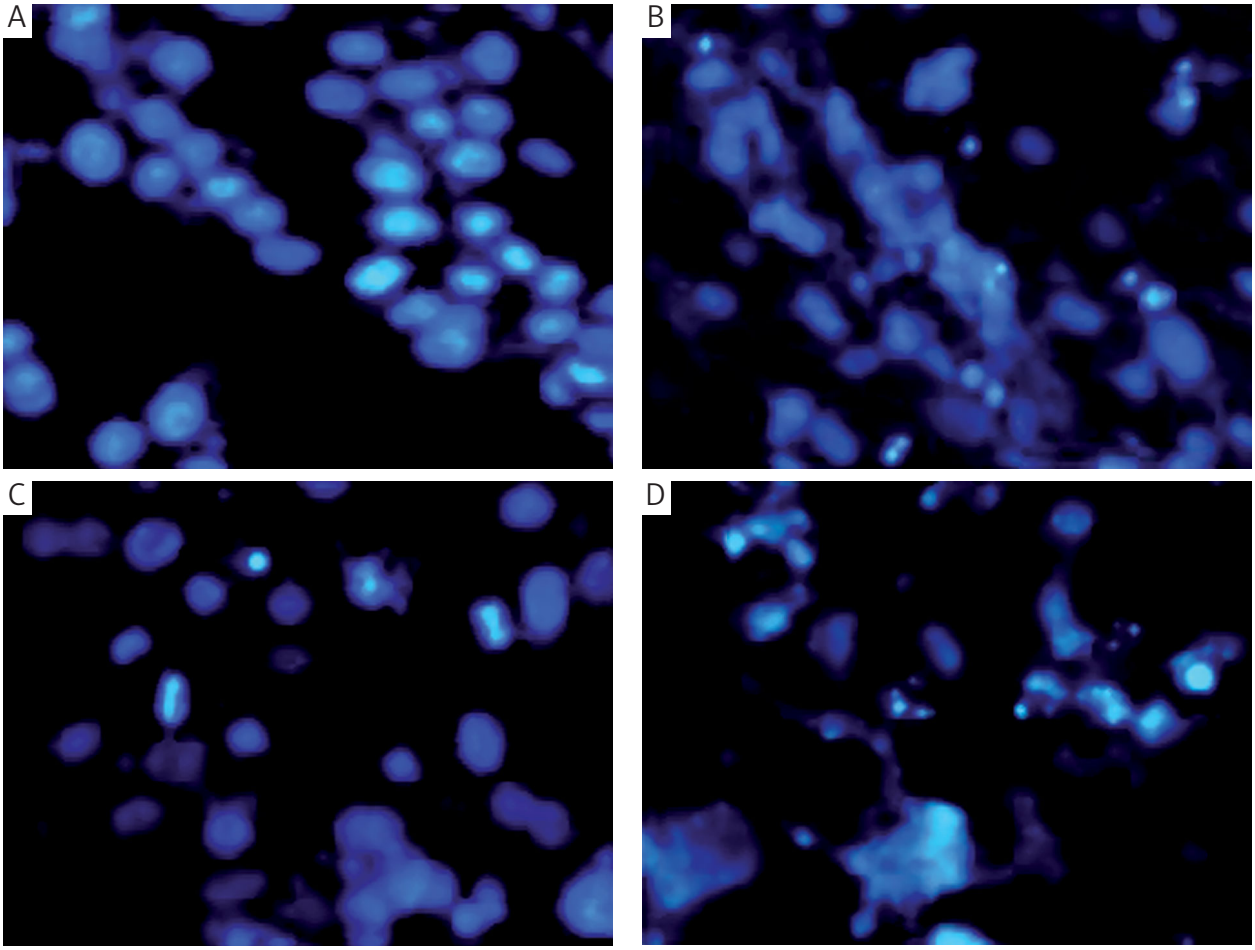

Fig. 4. Evaluation of apoptosis in T24 cell culture by DAPI staining. A – non-transfected cells; B – non-transfected cells treated with etoposide; C – cells transfected with shRNA/STAT3 construction; D – cells transfected with shRNA/STAT3 construction and treated with etoposide

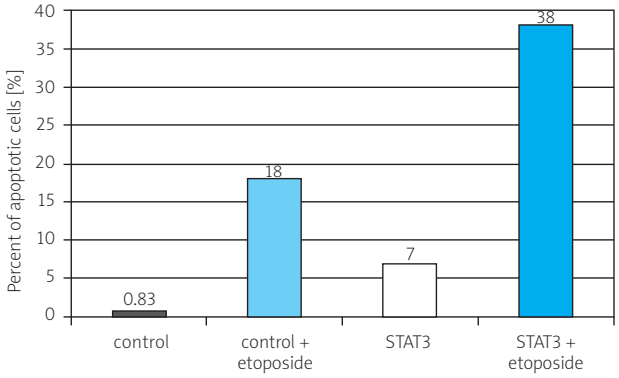

Fig. 5. The percentage of apoptotic cells in T24 cell culture estimated by measuring the level of DNA fragmentation using the Cell Death Detection ELISA Plus Assay

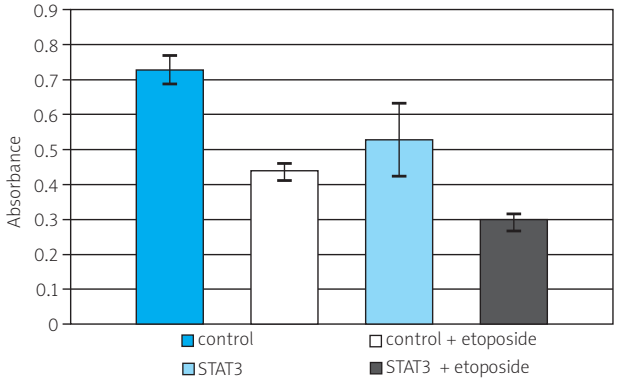

Fig. 6. Decrease in the absorbance signal connected with reduced proliferation rate in MTT assay conducted in T24 cell culture

staining, the largest percentage of apoptotic cells was observed in the group of cells transfected with the *STAT3*/shRNA construction and treated with etoposide (Fig. 5). The differences in the absorbance obtained in the MTT assay for each examined group allowed us to quantify the changes in cell proliferation. As shown in Fig. 6, the proliferation rate of cells with silenced *STAT3* in combination with etoposide was decreased approximately 2-fold compared to the untreated control. In both cases, statistically significant differences in cell apoptosis induction and proliferation were observed (respectively: $p = 0.0376$ for apoptosis induction and $p = 0.0318$ for changes of proliferation level).

Discussion

Activation of *STAT3* contributes to activation of several genes such as matrix metalloproteinases *MMP-2* and *MMP-9*, cyclin D1, VEGF and survivins, cytokines (including IL-6), chemokines and cyclooxygenase 2 (COX 2). Increased signaling through the *STAT3* proteins and related abnormalities can promote the development of neoplasm [18, 19].

Until now, the strategies of blocking *STAT3* protein function include the tyrosine kinase inhibitors [20–23], attempts of using antisense oligonucleotides which can block *STAT3* protein synthesis [24, 25], and RNA interference [26].

Many publications show the constitutive activation of *STAT3* proteins in etiopathogenesis of many malignancies, for example: hypernephroid carcinoma, papillary renal carcinoma, urothelial carcinoma, prostate cancer, lung cancer, ovarian cancer, pancreatic cancer, head and neck malignancies, leukemias and malignant melanoma [27–29].

Increased signaling with STAT proteins leads to proliferation stimulation and suppression of apoptosis. It is connected with constant stimulation of tyrosine kinases joined with the receptors for growth factors and cytokines (for example SRC, JANUS kinases, BCR-ABL, receptors with inner kinase domain) [30, 31].

Within earlier mentioned types of malignancies, urinary bladder cancer is one of the most frequent. What is more, it often recurs and it readily gives rise to metastases [32]. There is a high correlation between the overexpression of *STAT3* protein and the process of carcinogenesis. As a result, there is a need to find a therapy in which we can silence that overexpression.

Within the last decade, the post transcriptional silencing of gene expression has been widely discussed in the context of applying it in the diagnostics and therapy of many illnesses, including malignancies. Post transcriptional silencing of gene expression can be developed by antisense oligonucleotides, ribozymes or small interfering RNA molecules [20–26]. RNA interference (RNAi) can be achieved by applying two different kinds of RNA molecules: synthetic small interfering RNA (siRNA) and short RNA with hairpin conformation (shRNA) synthesized in an expression vector brought into the cell. Despite the fact that both siRNA and shRNA are able to create specific gene silencing, they show different silencing effectiveness. What is more, after induction from the vector, shRNA molecules cause lower, non-specific effects within genes that are not targeted. This difference is probably caused by the different mechanisms of including siRNA and shRNA in the RNAi pathway. If using a vector with insert coding shRNA (under the control of polymerase II), the original transcript is polyadenylated. Apart from that, siRNAs need to be used in higher concentrations than shRNA molecules if we desire effects at the same level. This makes siRNA molecules more susceptible to causing non-specific effects. What is more, unprotected siRNA can be degraded in the physiological processes [33].

To sum up, shRNA molecules have advantages which make them more useful and more effective than siRNAs, such as lower frequency of unspecific effects, the ability to silence a larger number of genes without a need to increase its concentration, durability of the effect and the ability to create the induced expression. For this reason, in the present work we silenced the expression of a gene coding the *STAT3* protein using an expression vector which contained and expressed the insert including the desired *STAT3*/shRNA molecules.

24 hours after transfection of the T24 cell line with the expression vector a decline of the mRNA level was observed. It was about 68% of the initial level in the examined cancer cell line T24. The investigations conducted by Rivat and co-workers show a similar decline of the *STAT3* expression to ours (approximately 28%) [34]. However, their investigational model was related to the large bowel cancer cell line HCT8/S11 and their siRNA sequence was binding to the *STAT3* mRNA fragment in the region from 1571 to 1589 nucleotides.

For silencing *STAT3* expression, Lifang and co-workers used three siRNA constructions, which bound to the following regions: from 1571 to 1580 nucleotides (first construction), from 438 to 456 nucleotides (second one) and from 2144 to 2162 nucleotides (third construction) of *STAT3* mRNA. The authors assessed the *STAT3* mRNA expression level 72 hours after transfection, using the Northern blot method. For the second construction, the mRNA expression decreased to the level of 25% and for the third construction to the level of 20%, related to the control culture. However, for the first construction, no silencing effect on *STAT3* mRNA expression was observed. Based on both the obtained results and analysis of secondary structure in bindings regions for *STAT3* mRNA, the authors advanced the hypothesis that silencing *STAT3* gene expression using siRNA constructions is dependent on localization of the siRNA sequence binding region in the target mRNA, and in consequence, possible secondary structures forming in this region.

In the present work, out of all suggested (by the siRNA program) interfering siRNA sequences, we chose the sequence targeting the mRNA fragment which includes the DNA binding domain (DBD). It is a key region for *STAT3* protein proper function.

Although the changes in the mRNA level were not so noticeable, the desired effect of the RNAi strategy with reference to changes of phenotype of cancer cells was observed. Namely, a statistically significant increase in the amount of apoptotic cells after etoposide stimulation was observed in cells with the silenced *STAT3* gene. That is the *STAT3* transcriptional factor which activates genes coding antiapoptotic BCL-2 protein, vascular endothelial growth factor (VEGF) or antiapoptotic survivin protein. Finally, it blocks expression of the gene coding P-53 protein, which is a very important effector in the process of apoptosis [35].

As the induction and the course of the process of apoptosis bring fast changes in the cells, we can suppose that even a transient and short-lived silencing effect on *STAT3* expression can bring the expected effect – increased elimination of cancer cells.

The sequence designed in the present work is effective, but the effect of silencing *STAT3* expression does not last long and its efficiency is low. This may be caused by cancer cells' "defense", used when they are denied of *STAT3* influence and when the expression of shRNA-*STAT3* construction is switched off, for example in the epigenetic pathway. In order to increase the efficiency of silencing *STAT3* expression in cancer cells and obtain the most profitable therapeutic results, it is worth verifying further shRNA-*STAT3* constructions designed for other localizations within *STAT3* mRNA.

The designed and synthesized genetic construction coding and expressing shRNA interfering molecules for the target transcript, mRNA for *STAT3*, shows the ability to suppress expression of the gene coding the STAT3 transcriptional factor. Along with decreased transcriptional activity of the STAT3

gene, the induction of apoptosis and decreased proliferation of modulated urinary bladder cancer cell line T24 can be observed.

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